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BIOLOGICAL BULLETIN

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VOLUME 143
JULY TO DECEMBER, 1972

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

The Biological Bulletin is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $5.00. Subscription per volume (three issues), $14.00.

Communications relative to manuscripts should be sent to Dr. W. D. Russell-Hunter, Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between May 23 and September 1, and to Dr. W. D. Russell-Hunter, P.O. Box 103, University Station, Syracuse, New York 13210, during the remainder of the year.

Second-class postage paid at Lancaster, Pa.

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PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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II. ACT OF CORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL] HENRY B. PIERCE,
Secretary of the Commonwealth

III. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised February 11, 1972)

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and nine Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-six Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed.

(B) Trustees *ex officio*, who shall be the Chairman, the President, the Director, the Treasurer, and the Clerk.

(C) Trustees *Emeriti*, who shall be elected from present or former Trustees by the Corporation. Any member of the Corporation in good standing who has attained the age of seventy years, or has attained the age of sixty-five and has retired from his home institution, and who has served a full elected term as a regular Trustee, shall be designated Trustee Emeritus for life at the next annual meeting provided he signifies his wish to serve the Laboratory in that capacity. Any regular trustee who qualifies for emeritus status shall continue to serve as Trustee until the next Annual Meeting whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation. The Trustees *ex officio* and *Emeriti* shall have all the rights of the Trustees, except that *Trustees Emeriti* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them except those chosen by the members, at any time. They may fill vacancies occurring in any manner in their own number or in any of the officers. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These bylaws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the bylaws will be acted upon.

RESOLUTIONS ADOPTED AT TRUSTEES' MEETINGS

EXECUTIVE COMMITTEE

I. RESOLVED:

(A) The Executive Committee is hereby designated to consist of not more than ten members, including the *ex officio* members (Chairman of the Board of Trustees, President, Director and Treasurer); and six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term. (August 11, 1967).

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice President. A majority of the members of the Executive Committee shall constitute a quorum and a majority of those present at any properly held meeting shall determine its action. It shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine. (August 12, 1966).

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by the Board or by law. (August 16, 1963).

(D) The Executive Committee shall keep appropriate minutes of its meetings, and its action shall be reported to the Board of Trustees. (August 16, 1963).

II. RESOLVED:

The elected members of the Executive Committee be constituted as a standing "Committee for the Nominations of Officers," responsible for making nominations, at each Annual Meeting of the Corporation, and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk). (August 16, 1963).

IV. REPORT OF THE DIRECTOR

To: The Trustees of the Marine Biological Laboratory

Gentlemen:

This report to the Trustees of the Marine Biological Laboratory is made at a time when past programs are being reviewed, when the Laboratory's objectives and the means earlier employed in seeking them are being restudied, and when the basic plans by which the Laboratory's scientific work is carried on are being reformulated.

The reassessment of objectives and procedures should be a continuing element in the conduct of any scientific institution; it has been such throughout the history of the Laboratory. But as Vannevar Bush wrote about another institution at a watershed, only rarely indeed do circumstances so shape themselves that the re-evaluation becomes itself a primary undertaking extending practically throughout the Laboratory.

This rigorous evaluation of the several programs by which, taken as a whole, the Laboratory carries out its mandate, is now in its second year. Thus far three general observations have emerged. First, we rightly take from this study renewed confidence in the central philosophy of the Laboratory. Secondly, it is clear that our scientists respect and draw heavily upon the resourcefulness of the Laboratory's able and loyal administrative and technical staff. Thirdly, our traditional organization is out of step with the realities of science support today.

The first two conclusions are reassuring. It is the third observation that gives cause for continuing concern. Our pattern of organization which in the past, and to a large extent, even today, has proved to be highly effective in providing opportunities for the productive investigator and promising student, in making available resources to leaders of proved skill in limning the unknown, may no longer provide adequate mechanisms for maintaining the financial viability of the Laboratory.

From the very beginning the Laboratory has sought the exceptional man and the exceptional program. Until recently our ability to compete for federal grants for graduate education and for institutional support made it possible for the Laboratory, at least to a very large extent, to march to its own drummer. Now the inability of the responsible federal agencies to respond to our proposals in these categories and the gradual restriction of their mandate to contracts and grants for circumscribed projects make it essential that we educe new ways of attaining our objectives in the face of ever-changing and ever-more restrictive federal guidelines.

Is our organization prepared to respond rapidly to changes required as new programs unfold? If not, what improvements should be made (and at what cost)? Which programs are most urgent? Which are likely to be most productive? Which have served their purpose? These are the principal questions to which the Officers and Executive Committee have addressed themselves during the year. In this review they have worked closely with four committees.

The mission of the *Finance Committee* is to reinforce the Executive Committee by providing a thorough assessment of the budget and the measures required to improve our financial situation. Its members are Robert Allen, Alexander T. Daignault (Chairman), William T. Golden, Samuel Lenher and John W. Moore.

The *Committee on Winter Operations*, chaired by George Holz, was asked to assess the current performance of the Laboratory in winter operations and to recommend changes required if and when those operations are increased. Its roster included George H. Clowes, Sears Crowell, A. L. Gorman, Clifford Harding, Robert Josephson, Robert Kahler, Hans Laufer, Yoram Palti and John Valois.

Upon Edgar Zwilling's untimely death, Eric Ball assumed the chairmanship of the *Salary Review Panel*. Despite his own illness and the sensitive nature of the assignment, he and his colleagues (John Arnold, M. R. Carriker and Catherine Henley) submitted a reasoned, thoughtful report which has already led to significant changes. The Panel itself has been discharged but arising out of it are two new bodies, a continuing *Committee on Employee Relations* and an *ad hoc Retirement Committee*. The former group includes three employees, Edward Bender, Robert Gunning and John Valois (Chairman) and three members of the Corporation (Lucena Barth, Catherine Henley, Ivan Valiela, serving one, two and three year terms, respectively). As vacancies occur by rotation, employees will elect their own representatives; the Corporation representatives will be appointed by the Director.

John Arnold (Chairman), Gertrude Hinsch and J. W. Lash constitute the *Retirement Committee*, which has been asked to recommend improvements in the Laboratory's pension program which might be put into force by January 1, 1973.

This year, as last, I have placed emphasis on contributions arising from interactions of many Corporation members and employees. From such cross-linkages we may expect added effectiveness, providing their recommendations are regarded, not as rigid prescriptions, but as signals to the Trustees who are the arbiters of our long-range policy.

It is against this background that I shall summarize the principal actions and decisions of the past year.

The Laboratory as a year-round center

Although our proposal submitted to the National Science Foundation on May 12, 1971 has not been officially declined at this writing, we have been informed that regulations of the Office of Management and Budget currently in force prevent the Foundation from providing institutional support or from considering the Laboratory as a modified national laboratory, as discussed by our Executive Committee with members of the Foundation's staff. We have been advised that the Foundation is prepared to evaluate research proposals from individuals and teams, but that it cannot provide the "primer" funds called for in our proposal to equip and staff the Laboratory for year-round research. One of the actions taken by our Trustees at their meeting on February 11, 1972 has an important bearing on the question. In approving the Executive Committee's resolution that a Deputy Director be appointed to be in residence throughout the year, the Trustees expressed their confidence that, providing someone with the requisite breadth of vision in research and qualities of leadership can be found, a research nucleus can be established that will ultimately act as a focus for the development of a series of related programs. The identification of such a "chief scientist in residence," working closely with the Director and General Manager should permit such a division of labor and distribution of responsibilities as to insure a more effective overview of the Laboratory's needs.

At the same time, we are attempting to attract small groups of highly competent investigators to the Laboratory. By emphasizing groups, we do not mean to categorically exclude the lone independent scientist. However, we do wish to stress in year-round programs, as we do in the summer, the advantages of exchange, of combining leaders of proved acumen and promising younger scientists, of balancing and combining the knowledge of several investigators.

In addition, more than in the past, we shall try to carry on a continuing review of our year-round research. Some studies carried on for considerable periods in relative isolation appear to have reached the point where they should be carried forward by other agencies in locations where they can profit by more frequent peer review.

It may appear paradoxical that while attempting to strengthen year-round research (including environmental biology), we have announced the termination of the Systematics-Ecology Program at the close of its first decade. The announcement was not made without regret, for not only was the concept of the Program as initiated by Philip Armstrong and Arthur Parpart bold and, in its day, forward-looking, but especially in its formative years the Program contributed significantly to the Laboratory's vitality. However, during the past few years its financial fortunes have steadily declined to the point at which no other decision appeared possible. I shall not enumerate the Program's many contributions, which its Director, M. R. Carriker will treat in his final report, nor shall I attempt to educe the reasons for its failure to attract funds, except to observe that during the period when the support of systematics was waning and of environmental biology waxing full, the Program's emphasis and strength tended to lie more on the side of systematics. It may also be true that, as one reviewer put it, "Systematics is scarcely more allied to ecology than to developmental biology."

Winter teaching has been limited almost entirely to the Boston University Marine Program. During the second semester, 1971–1972, four staff members were in residence, the Director, Arthur Humes, William Stewart and Ivan Valiela being joined for the term by Stephan Golubic, teaching Marine Microphytes. Fourteen graduate students and four seniors participated for all or part of the year. At least 120 inquiries about graduate study have been received for the year beginning in September 1972.

Beginning in January 1972, the first two undergraduate students of the Consortium of Northern New England arrived for the second semester. Five colleges and universities offered formal courses at the Laboratory in the fall and spring, 1971–1972: Davidson College, Drew University, Oakland (Michigan) University, Temple University and University of Michigan. Three institutions conducted special programs for their students: Massachusetts Institute of Technology, University of Notre Dame, and Woods Hole Oceanographic Institution.

The year also found a number of pre-college students using the Laboratory's facilities, space having been made available to the Falmouth school system for students in advanced biology and in a marine sciences program.

The summer courses: an agonizing reappraisal is necessary

The quality of our summer offerings has rarely, if ever, been higher. At the close of the summer of 1971 we bid adieu to three Instructors-in-charge, James F. Case (Experimental Invertebrate Zoology), Andrew Szent-Györgyi (Physiology) and Malcolm Steinberg (Embryology), all of whom served responsibly and with distinction as leaders of innovative courses. In their places starting in 1972 are Robert Josephson, John Cebra and Eric Davidson. Of the three, only Josephson, now at the University of California, is a member of the Corporation, known personally to most readers of these Reports. Cebra, a distinguished immunochemist-cell biologist, Professor of Biology at Johns Hopkins, is a newcomer; while Davidson, who, like Cebra, was trained at Rockefeller University, and is now a faculty member at California Institute of Technology, will be starting his second summer at the Laboratory.

New leaders have also come forward to direct the Research Training Program in Excitable Membrane Biophysics and Physiology, David E. Goldman of Woman's Medical College of Pennsylvania and John W. Moore of Duke University having succeeded W. J. Adelman who had guided the program since its inception.

Having remarked upon the quality of our courses and some of their leaders, it seems incongruous to report that nearly half of the Laboratory's nine courses and training programs are operating in 1972 without sufficient funds.

Four programs have federal funds. The Embryology course and the Research Training Program in Fertilization and Gamete Physiology are supported by grants from the National Institute of Child Health and Human Development; the Physiology course continues to draw its funds from the National Institute of General Medical Sciences, and our program in the neural sciences for minority ethnic groups, Frontiers in Research and Teaching, is assured of three years' support from the National Institute of Neurological Diseases and Stroke.

A generous gift from Dr. Ernest B. Wright, supplemented by a grant from the Alfred P. Sloan Foundation, has permitted the continuation of the Program in Excitable Membrane Biophysics and Physiology. Neurobiology can be continued thanks to another gift from the Grass Foundation which has played so vital a role in training in the neurosciences at the Laboratory. However, it is essential that major support be found from other sources. The National Science Foundation has denied support to Ecology, Experimental Invertebrate Zoology and Experimental Marine Botany. Fortunately the Research Corporation has made a special award in support of an innovative program of faculty-student research in experimental marine ecology. Zoology and Botany will be offered in 1972, with the Laboratory providing minimal essential funding.

Our proposals were declined because recommendations for their support would be contrary to the National Science Foundation's policy not to support teaching with research funds. Although there are large components of research in our proposals, with at least half the time of both staff and students being devoted to research, there is no denying that training is a primary objective.

There is, in my judgment, a continuing need for such cousres in national centers like the Laboratory; courses like these are vital for maintaining the depth and robustness of science throughout the nation. The current policy at the National Science Foundation (which is, I believe, inimical to the Foundation's charge) will result in the well being of our science being left in the care of a community of aging scientists, with an attendant deterioration in innovation. The Foundation's policy denies the need for renewal.

I am not alone in objecting to this short-sighted policy; nonetheless I believe we must assume that it will not be reversed in the immediate future. Hence the heading of this section: an agonizing reappraisal.

All of the facts are not yet in. The fundamental question is this: How will the complete lack of funds for student stipends, travel and tuition affect both the quality and numbers of students enrolled in these courses? The Laboratory cannot continue to fund these courses, even at a minimal level. They must be "self"-supporting; here I use "self" to mean that they must attract funds from a source other than the Laboratory itself. Even if we assume that the courses would be fully subscribed, with students (or their colleges and universities) willing to pay their fees, they would not be self-supporting at present levels of tuition, which are already high.

In the weeks immediately ahead, the Officers and Trustees, the Instruction Committee and Instructors-in-charge will have to find a solution. Some of the possibilities that have already emerged are the following: (1) The abolition of courses as they are presently constituted, with the development of summer research "program-projects" involving graduate students as research participants. It is a moot question whether such an approach could be funded. (2) A change in the character and structuring of the courses (possibly combining two or more), with a substantial reduction in the number of instructors; in short—if sufficient paying students can be recruited—to operate one or more courses on a break-even basis. (3) An even more drastic change in the character of our offerings—for example we might offer refresher courses to college or high school teachers.

At the risk of biasing future discussions, I would emphasize that I lean away from any solution in which we opt for mere survival—a course for the sake of having a course. We have been pathfinders in graduate research training; we must maintain our resiliency and find new ways of remaining in the vanguard.

Support of the CAP'N BILL

The National Science Foundation has approved our request for $15,000 per year for three years, beginning in 1972, for our project, "Support of a charter vessel for collecting materials for research in neurobiology."

This grant will provide about half the cost of chartering the CAP'N BILL, the remainder to be obtained as in 1971, through a surcharge imposed on investigators using the ship's services. However, the grant will insure that students and younger independent investigators who lack grant funds to cover such charges may have full access to squid and other animals provided by the CAP'N BILL.

Annual giving

In the summer of 1971 the Laboratory initiated its Annual Giving Campaign, patterned after the annual "rollcalls" of colleges and universities, and successful fund-raising campaigns at our sister institutions, for example the Jackson Laboratory and the Cold Spring Harbor Laboratory. The idea did not meet with *complete* approval. At least a few members of the Corporation registered their concern that—to put it as bluntly as one critic did—an investigator might "buy his way into the Laboratory." Nevertheless, most of our members realized that it is a fact of life that we—all independent laboratories—are more dependent than ever on the help received from our local neighbors, our scientific alumni, foundations, and most importantly, our own members. We must assign the Laboratory a high priority in our own giving.

In the first year of the program, 177 Corporation members contributed and pledged (through May 18, 1972) $76,908. The Campaign will be renewed in October, 1972, and each October thereafter. Hopefully we will continue to merit the backing of those who have contributed in the past as well as of many new friends.

MBL *Award*

In the summer of 1971, a generous gift from an anonymous donor enabled the Laboratory to establish a prize to be known as the MBL Award, to be given for a noteworthy paper presented annually at the General Scientific Meetings. The monetary value of each award will be $100.

It is a condition of the award that to be considered eligible the work must have been carried out at the Laboratory. Ordinarily the presentation at the General Scientific Meetings will have been the first public presentation (apart from discussions at departmental seminars and the like). Although some preference will be given to junior investigators, it is recognized that occasionally established scientists have new ideas too. Accordingly no nominee will be excluded on the grounds of age.

Judging will be based not only on the material presented at the Meeting but on a manuscript to be submitted by the nominee. Following the Meetings the chairmen of sessions, or any other listener, may submit in writing a nomination to the Director. Nominees will then be asked whether in their opinion their work meets the established criteria. If the paper is eligible, the nominee will then submit to the Director within four months following the meeting a description of the principal findings not to exceed 1500 words, together with any other supporting data he sees fit to include.

The Committee of Judges had a difficult time in selecting the first winner, so difficult in fact that it has decided upon a dual award: to J. E. Lisman and J. E. Brown for their paper, "Effect of intracellular pressure injection of Ca^{++}-EGTA buffers into *Limulus* ventral photoreceptors" and J. R. Whittaker, whose contribution was entitled "Differentiation without cleavage in an ascidian egg: Development of muscle acetylcholinesterase."

A loss—and a gain

On August 13, 1971, Gerard Swope, Jr., announced his retirement as Chairman of the Board of Trustees. On that occasion I read a statement prepared jointly by P. B. Armstrong, H. B. Steinbach and myself. It read, in part,

"A man's career is usually reckoned in terms of his achievements; by that reckoning Jerry's leadership has been exceptional. For nearly two decades we have benefited from his wisdom, two decades marked by material progress, by new buildings, and by new programs, all of which have been advanced with a very keen eye toward preserving the high ideals of the Laboratory. Possibly it is unfair to single out one achievement for special mention, but his leadership at all stages in the development of the dormitory-dining hall was truly exceptional. However, the picture of Jerry Swope shines with a special luminosity that scintillates not just from these definitive symbols of progress, but from his unique combination of personal qualities—his warmth and kindness and his understanding of the Laboratory and its people. He has understood the lively, independent mind and the factors necessary for their interaction. He has worked toward the evolution of a community receptive to new ideas.

Jerry has helped to train, and has guided three directors, all of whom have been pleased to serve under his stewardship. No director could have hoped for a more productive association.

Jerry has provided wisdom and practical advice, occasionally with a disconcerting ability to isolate weak points for scrutiny in the light of legal logic. We owe very much to Jerry Swope and as recipients of his guidance in the past we hope to lay claim to his continued wisdom in the future. To that end, however, we must take steps to provide the opportunity, for "In a busy life no convenient time ever comes to go visiting in cold blood. Some pragmatic stimulant is necessary." Boswell observed of his hero, Dr. Johnson, " . . . on clean-shirt day he went abroad and paid visits." Therefore the Trustees have this day elected Jerry Swope Honorary Chairman of the Board of Trustees."

Many members of the Corporation became acquainted with our new Chairman, Denis Robinson, even before his election. His penetrating questions at the Symposium on National Policy and the Life Sciences and his lively participation in a wide range of Laboratory affairs provided evidence of his understanding of the role and needs of the scientific community in Woods Hole. For those who may not have an immediate opportunity to get acquainted with him, here is a "thumbnail sketch." One of the nation's most distinguished electrical engineers (Ph.D., University of London), he worked in laboratories at the University of London, MIT and the University of Birmingham (England) until 1946 when he founded, and for 24 years served as President of, High Voltage Engineering Corporation, where he is now Chairman of the Board. Among his many honors, he is a Fellow of the American Academy of Arts and Sciences, the American Physical Society and the Institution of Electrical Engineers (London), and a member of the National Academy of Engineering. His association with the Laboratory is especially timely because of his deep concern about the recycling of wastes as they affect our water resources, and with problems arising out of the siting of power plants. Denis and his wife Alix are "nearest neighbors" to MBL Beach, their summer home being located on Gosnold Road.

The flow of energy

In a tribute to J. Walter Wilson, Henry Wriston remarked that Wilson "did not look to the president to supply all the energy." Reflecting on his own years of service as President of Brown University he observed that the office is, inescapably, an energy post, and spoke of the "corporate inertia" of the faculty. In a large and complex organization like the Laboratory, not all the moving vigor can flow ceaselessly from the Director. It needs to be generated and manifested at every level. Perhaps the most disturbing consequence of our failure to attract major institutional support is that younger investigators may find it increasingly difficult to come to the Laboratory. We must increase the number of promising young scientists in our midst, and open channels for their participation in our affairs. We must look to tomorrow's contributors, who will keep us abreast of the host of novel challenges that will surely occupy the Laboratory in the future.

I would close with a thought I expressed on another recent occasion. It is especially fitting in this context.

Not long ago I had the privilege of contributing one of two prefaces (the other contributor being Peyton Rous) to Paul Weiss's *Dynamics of Development: Experiments and Inferences*. I often think that every critical review and every annual report should have authors from two generations. *We see what we are conditioned to see.* My glimpse of the Laboratory tomorrow is only one of many possible visions.

In *Between Pacific Tides*, John Steinbeck put it most eloquently: "There is in our community an elderly painter of seascapes who knows the sea so well that he no longer goes to look at it when he paints. He dislikes intensely the work of a young painter who sets his easel on the beach and paints things his elder does not remember having seen."

1. MEMORIALS

HAROLD SELLERS COLTON

By WILLIAM F. DILLER

Dr. Harold Sellers Colton, Emeritus Professor of Zoology at the University of Pennsylvania, died at Flagstaff, Arizona on December 29, 1970. He was a native of Philadelphia where he was born on August 29, 1881. He received his B.A. degree from the University of Pennsylvania in 1904 and his M.A. in 1906. He then became a student of two distinguished members of the zoology staff, Dr. J. Percy Moore and Dr. E. G. Conklin. Under their guidance he completed a doctoral thesis entitled "Some effects of environment on the growth of a pond snail *Lymnaea columella* Say," published by the Academy of Natural Sciences in Philadelphia. He was awarded the Ph.D. degree by the University in 1908. Dr. Colton was in the summer course in zoology at the Marine Biological Laboratory in 1905, was elected a member of the corporation in 1908, and retained his interest in MBL into his retirement.

With the exception of two years (1918–1919) in the Army, where he served in the Intelligence Service with the rank of Captain, he continued his association with the Department of Zoology at the University of Pennsylvania, and served for a number of years as head of the course in elementary zoology. In 1926, as the result of the death of a son, he moved to Arizona. Here he threw himself with enthusiasm into studies of the geography, geology and anthropology of northern Arizona, as well as into participation in many civic projects and organizations. Adjacent to his home he established a small laboratory known as the San Francisco Mountain Station of the University of Pennsylvania, devoted to the ecology of the local fauna.

At the University of Pennsylvania he is perhaps best known for his outstanding service as director of the course in introductory zoology, where he made many innovations; and as the author of publications on educational statistics. Those who, like myself, were privileged to work under his direction as young instructors in the elementary course have remembered him through the years as an outstanding educator and as a charming, energetic and stimulating leader whose example has been an inspiration throughout their own teaching careers. His research interests during his active tenure at the University included the ecology of fresh water and marine molluscs and excretion in Ascidians.

Dr. Colton's wife, the former Mary Russell Ferrell, was a noted Philadelphia painter (one of the so-called "Philadelphia Ten"). Both became greatly interested in encouraging Indian arts and crafts and in the prehistoric ceramics of the area. Together with her, Dr. Colton founded the Museum of Northern Arizona in Flagstaff, and served as its Director from 1928–58, continuing as Director Emeritus until his death. He is credited with a major part in the founding of craft shows to encourage Indian artists, and with the establishment of two National Monuments, Sunset Crater and the Wapatki Ruins north of the Crater. He was one of the first scientists to realize that the volcanic area had been occupied by prehistoric Indians prior to massive eruptions in 1064–67. His efforts led to the preservation of one of the richest archaeological sites on the North American continent.

Dr. Colton was appointed to the staff of Arizona State College in 1926, where he held a professorship until 1953, and an Emeritus Professorship from 1953 until his death. He was the recipient of the LL.D. degree from the University of Arizona in 1955, and the D.Sc. from the Arizona State College in 1958. He was a member of many important societies and was the author of more than 240 articles, monographs and books on widely varied subjects. His writings include reports on marine zoology, insect morphology, museum history, and on the archaeology, geology and atmospheric conditions of Northern Arizona.

He is survived by his wife, one son, Captain J. Ferrell Colton, two granddaughters, and four great-grandchildren. Also surviving are two sisters, Mrs. Robert T. Wilson of Tucson, Arizona and Mrs. Robert P. Esty of Ardmore, Pennsylvania.

MANTON COPELAND

By P. Sears Crowell

Manton Copeland was born July 24, 1881 in Taunton, Massachusetts and died at his home in Brunswick, Maine, May 22, 1971. He received his degrees from Harvard, the Ph.D. in 1908. He married Ruth Winsor Ripley in 1910. His dedication to teaching at Bowdoin College from 1908 until retirement in 1947 and his influence on students and through them on biology and medicine is attested in many ways. When Dr. Alfred C. Redfield was in charge of Zoology at Harvard, he looked into the question of where their graduate students came from and found that Bowdoin had sent more in Zoology than any other school, including Harvard College. In 1960 former students initiated a memorial scholarship fund with a goal of $25,000. Over the years contributions of students and also many Woods Hole friends and others built "Fundy," as it was named by "Copey," to the hoped for amount. This was a great satisfaction to him and reflects the affection of many individuals. Twelve Copeland scholarship awards have already been made. During most of his years at Bowdoin, when he was head of the Department of Biology, he taught general zoology as a year course, a semester course of general botany, and a course called "Genetics and Evolution" popular with non-majors as well as biology students. Besides teaching two courses and their laboratories each semester, he usually had one or more students carrying on a research project.

The citation of the Bowdoin Alumni Council's Alumni Award for Faculty and Staff, given in 1966, reads in part: "A citizen of Brunswick, a summer resident of Woods Hole on Buzzards Bay, and a collector incarnate of moths, butterflies, worms, sewing birds, duck decoys, flowers, books, friends, students, and children." All were cared for meticulously. In the case of the sewing birds he produced a taxonomic key.

He worked at Woods Hole as an investigator, first at the Fisheries, and from 1915 to 1945 at MBL. He was elected to the Corporation in 1913. His studies dealt with speciation in mammals, physiology of mollusks—particularly ciliation and chemoreception. Probably his most interesting experiments were those in training *Nereis* (*Neanthes*). He demonstrated conditioned reflexes (in the Pavlovian sense) in these worms. He was a member of the Society of Mammalogists, Ecological Society of America, American Society of Zoologists, American Ornithologists Union, and a Fellow of The American Academy.

In the Woods Hole community he was perhaps best known for his home, "The Roost," on the hill north of the bathing beach and the development with the aid of his sons of a delightful labyrinth of paths leading to little special gardens, overlooks, and rock pools. His three sons and daughter with their families have spent part of each summer at "The Roost" and its annex, "The Coop." One of "Copey's" delights was to conduct guests through the paths and constructions, perhaps ending with the view from the deck of the "Artemisia," his secluded study at the hill's crest, which was fitted out like a ship's cabin, or "The Copecabana," a shelter and picnic area above the rocky shore. There a posted sign reads:

> "The real purpose of The Copecabana is not for the sipping of cocktails, or the cracking of lobster shells. Rather it is to give you some protection from the elements and a feeling of seclusion that you may better appreciate and gain more happiness from the everchanging picture that lies before you. Your are in one of the most beautiful and exciting spots on earth—where the land meets the sea. Here life is probably more abundant than in any other locality; in fact, it may have arisen here. The littoral is never the same. It varies with every tide, wave, and wind. . . . Come here alone, or with an understanding companion, in the early morning, or when the sun is setting over Penzance. Look before you, in front of you, and above and try to understand what it all means. Come again and again because what you see is not all that you can see. Eventually you may reach the only possible conclusion. In any case you will dream better dreams, which is a key to happiness. And that is the real purpose of The Copecabana."

CHARLOTTE HAYWOOD

By Curtis J. Smith

It is never possible to capture the essence of a complex personality in a few words. If it *were* possible, the words used of Charlotte Haywood would sound like the exhortations of a slightly old-fashioned schoolmaster: "strength of character," "self-discipline," "absolute integrity," and "selfless devotion." No one would deny that Charlotte exemplified these virtues, but no one who knew her would allow that such phrases do justice to her liveliness and enthusiasm, her love of beauty, or the warmth of her friendship.

She was, in many respects, a fairly typical product of the great patrician tradition of New England. As a girl she often accompanied her physician father on his rounds in a horse and buggy. She must have absorbed the uncompromising standards of her Yankee heritage, along with the appreciation of art and the love of science which marked her whole life.

As an undergraduate at Mount Holyoke College she began an association with Abby Turner, who, along with Ann Morgan, carried on the traditions of excellence in science under the impetus of the legendary Cornelia Clapp. The association was to continue for the rest of Miss Turner's life; and it is one of the tragedies of Charlotte's own life that she was never able to find a protegée to continue this worthy succession.

Those undergraduate days were not all plain living and high thinking! Charlotte was always fun-loving and full of the zest for living which remained one of her most charming characteristics to the very end. Only a few years before her retirement she managed to hitch a ride on Otto Kohler's antique fire engine while it was transporting male members of the faculty on a careening tour of the campus during their annual post-graduation party. She later remarked, with considerable satisfaction, "It was something I've always wanted to do!"

Endearing as her personal traits were, it is in her career that one finds her greatest monument. Between earning her master's degree at Brown University and her Ph.D. at the University of Pennsylvania, she returned to Mount Holyoke as an instructor of physiology from 1921 to 1924. After receiving her doctorate she taught for three years at Vassar, but in 1930 she came back to Mount Holyoke for the career of teaching and research that was to continue for the rest of her life.

In that long span of time a great many of her students have achieved distinction in careers of their own. They provide visible testimony to the excellence of her teaching and the inspiration of her example. But even more than this, it was in her elementary courses, taught not specifically for science students, but as a part of a liberal education, that she made her greatest mark. Hundreds of students who got their first, and sometimes their only, taste of science at her hands, remember her with affection and respect; and few of them could have realized how meticulously the smooth-running laboratories were rehearsed with the teaching staff, or how many hours she spent preparing each lecture, no matter how familiar she was with the material. Even when the enrollment exceeded two hundred, she would know the name of every student in her class, and no returning alumna was unrecognized. There are many roads to immortality; and in the many hundreds of students that knew Charlotte Haywood her memory lives on.

Although her love for teaching took first place in her life, Charlotte was an active and productive research scientist as well. Many summers were spent at her beloved Woods Hole, at the marine biological station, carrying on her studies in respiratory physiology. Dr. Helen W. Kaan, her classmate and close friend, recalls that beginning with the summer of 1920, Dr. Haywood spent virtually every summer (except those spent in European laboratories) in research and teaching at the Marine Biological Laboratory in Woods Hole. After her retirement in 1961 she continued faithfully to attend the

Annual Corporation Meetings at the Marine Biological Laboratory, and was present at the August, 1970, meetings. She was one of the first woman members of the American Physiological Society, and was known and respected by a great many of the scientific "establishment." After her retirement she continued her work on her scientific publications, and attended seminars whenever she found time in her busy life.

Although her whole professional life had been characterized by an extraordinary devotion to her students and her work, upon her retirement Charlotte revealed new talents and found new outlets for her restless mind. Always an ardent traveler, she was finally able to extend her wandering to a trip around the world. Her life-long love of flowers was expressed in a new-found skill in photography. She continued her regular attendance at concerts, plays, and lectures; and she found more time for her friends.

Throughout her life her contacts with students, friends, and associates were characterized by an ingrained and spontaneous graciousness. She was uncompromising in her personal standards, but forgiving of others. Perhaps she typified her era, but her gentle nobility of spirit is a quality needed by every era. Her passing sadly diminishes our world.

FAITH STONE MILLER

By S. Meryl Rose

Faith Stone was born in Newton, Massachusetts on September the twenty-third, 1908 to Alaric Maxwell Stone and Ruth Taylor Stone. Hers was a full life as daughter, wife, mother, friend, research worker and teacher until her sudden death on June 11, 1971, in Falmouth, Massachusetts.

She grew up in and attended school in Newton. After graduation from Newton High School she entered Mount Holyoke College with a College Entrance Board Competitive Scholarship for New England. From her sophomore year through till graduation in 1930 she held an Alumnae Association Scholarship. During the next two years she served as an Assistant in Zoology at Connecticut College for Women. After that she went on to the University of Chicago where she received the Ph.D. in Zoology in 1937. It was at Chicago that the great collaboration between Faith Stone Miller and her husband, James A. Miller, Jr., began. They were both students of Charles Manning Child and began their studies on regeneration under him. One of the many things they observed was that decreased temperatures prolong life. Their research evolved into their well known studies demonstrating that hypothermia can cause recovery from asphyxia in the newborn.

There were some important years when most of her time was spent with her children, David and Janet. When they became self-sufficient she returned to academic work in 1948 as a part-time Research Assistant working with her husband at Emory University. There she also became the Director of Basic Sciences for Nurses. Then in 1960 came the move to Tulane University when her husband became the Chairman of the Department of Anatomy. She advanced from Assistant Professor to Associate Professor of Anatomy and became the recognized and respected unofficial Assistant Chairman. Together Dr. Faith Miller and her husband built an excellent department including an outstanding center for the training of anatomists. It was in her work with graduate students that Dr. Miller was at her best as a teacher. With quiet, kindly humor and sincere interest she brought out the best in them. She always had as much time as required for their academic and personal problems.

There were awards and prizes along the way. One of the early ones was the Collecting Net Award for Excellence in Invertebrate Zoology at the Marine Biological Laboratory. The laboratory, where she became a member of the Corporation, their home in Woods Hole and its environs grew in importance to her. She shared with her husband in 1959 the Southeastern Biologists Research Prize and the Sigma Xi Research Citation at Emory University. She was the Martha Catching Enochs Fellow of the American Association of University Women during 1957–1958 in London and in 1962 was a Fulbright Research Scholar in Finland. These years, including a period in Sweden, were spent with her husband on the valuable studies which established that infants asphyxiated during birth could be revived without subsequent ill effects. The results of the Millers' research were reported in many papers and at national and international meetings. Confirmation of the beneficial effects of hypothermia in overcoming asphyxia has come from investigators in several European countries.

Dr. Faith Miller was a member of the Society of Sigma Xi, the American Association of Anatomists, the American Society of Zoologists, the Association of Southeastern Biologists and the Southern Society of Anatomists.

Visits to foreign lands were opportunities for collecting beautiful old objects. Many of these are in their tastefully restored home in old New Orleans where she served as a gracious hostess. Dr. Faith Miller's unique blend of knowing kindness, quiet humor and personal interest in her associates has enriched the lives of many of us.

EMIL WITSCHI

By JOYCE BRUNER-LORAND

The life motif of Emil Witschi was his love of nature and his untiring and selfless devotion to scientific research. Professor Witschi was born in a small village near Bern, Switzerland, February 18, 1890. He spoke fondly of his early years spent in the pastoral surroundings of alpine valleys which offered him the luxury of contemplation of the many forms of developing life. By the time he went to Munich to pursue his doctoral studies under the guidance of Richard Hertwig, he was thoroughly indoctrinated as a naturalist. He received his doctorate in 1913, and published the work with which he was most intimately identified, the inductor theory of sex differentiation in 1914. From his studies on sex differentiation in *Rana temporaria*, he very early concluded that all embryos and the primordial germ cells are bipotential, that they may develop in either the male or female direction, that the alternative of male or female differentiation depended upon both genetic and non-genetic factors. His demonstration that the embryonic gonadal cortex acts as inductor of female differentiation and that the medulla induces male differentiation is basic to all considerations of the problem of human intersexuality.

Professor Witschi left Switzerland in 1926 and came to America as a Rockefeller Foundation Fellow. He had barely set foot in the United States when he was requested to deliver an evening lecture at the Marine Biological Laboratory, an event which he often recalled with a mixture of terror—speaking in a new language, and with pleasure—as it marked the beginning of association with the MBL which was to last almost half a century.

In 1927 Professor Witschi joined the faculty of the State University of Iowa. He was particularly fond of its beautiful setting amid the rolling hills of the Iowa countryside overlooking the Iowa river. It reminded him of his native Bernese Oberland. His devoted wife Martha and his two children joined him there and the decision was made to assume American citizenship. Few universities have been so privileged to count among its faculty a man of such outstanding and diversified achievement. He was a dedicated teacher. Those who attended Professor Witschi's embryology lectures can hardly forget his ability as an artist. The most intricate three dimensional drawings were swiftly and deftly executed. His lectures were truly a unique intellectual and artistic experience. His great knowledge in the field of embryology found expression in a textbook "The Development of Vertebrates," published in 1955.

In the laboratory, Professor Witschi's work covered a broad range of topics, pursued with endless enthusiasm, devotion and the highest degree of excellence. He understood the importance of comparative biology, and his work in endocrinology and sex differentiation was carried out on more than 30 forms, ranging from invertebrates to man. His contributions have been fundamental to the understanding of many fields of endeavor: human sex development—genetic, developmental and hormonal aspects of gonadal dysgenesis and sex inversion in man; the migration of germ cells in the human embryo; the comparative aspects of pituitary gonadotrophins; the evolution of endocrine reactions—hormonal control of feather and bill coloration in birds; teratogenesis and fetal abnormalities—overripeness and temperature effects in the amphibian egg; genetics—the mechanism of sex inheritance in amphibians; behavior—studies on the herring gull; underwater hearing—the basilar papilla of the amphibian ear.

Emil Witschi was the recipient of many honors. We can mention the Fred Conrad Koch Award and medal, the honor of highest distinction conferred by the Endocrine Society, and the degree of honorary Doctor of Medicine conferred on him by the University of Basel on the occasion of the 500th anniversary of its founding. He was doubtlessly pleased by these and the many other honors which came his way. But for Emil Witschi satisfaction lay in the inspiration he provided to his students, over 45 men and women who earned their Ph.D. degree with him, and to the many other researchers who by virtue of his example and encouragement would continue with the same vitality and dedication for which we shall always remember him.

For those of us who were privileged to enjoy the warm hospitality of his home so elegantly presided over by his gracious wife Martha, the memory will always be cherished.

EDGAR ZWILLING

By Mac V. Edds, Jr.

Edgar Zwilling was born in Pittsburgh on February 1, 1913; he died on July 23, 1971. His parents were Russian expatriates; his father, a blacksmith, lived only until Edgar was five. The family moved then to Brooklyn where Edgar grew up. He saw the prosperity of the 20's only from a distance; his boyhood was one of harshness, struggle, and hard-won reward.

Edgar attended Brooklyn College from which he was graduated in 1933; there he had developed an interest in biology, and he turned to graduate study in embryology at Columbia University where under the guidance of Lester Barth he received the Ph.D. in 1940. For the balance of his life, Edgar served three institutions with devotion and steadfastness: The University of Connecticut as an investigator in the Animal Genetics Department of the Storrs Agricultural Experiment Station; Brandeis University as professor and chairman of Biology; and the Marine Biological Laboratory as student, instructor, investigator, committee member, and tireless advocate. More briefly, Edgar also served the National Science Foundation as Program Director of Developmental Biology, the National Institutes of Health as a Training Grant Panel member, the Society for Developmental Biology as its president, the American Society of Zoologists as chairman of the Developmental Biology Division. One of his last honors came in election to the American Academy of Arts and Sciences.

As these marks of recognition attest, Edgar came to be held in high regard for his contributions to developmental biology. His forte was experimental morphogenesis practiced at tissue and organ levels. But he was no stranger to the cellular and molecular approaches that evolved during his academic life, and he used them skillfully to contribute understanding to the genesis of form at higher levels. Considering his nearly single-minded refusal to be distracted by merely fashionable lines of research, Edgar worked on a surprising diversity of developmental problems, including more or less in chronological order, the origin of the nose and the ear in the frog embryo, regeneration in hydroid coelenterates, etiology of developmental anomalies, and—his major work—cell and tissue interactions in the development of the chick limb. In now classical papers starting in the middle 1950's, Edgar analyzed the outgrowth of limb bud mesoderm under the influence of the apical ectodermal ridge, as well as the reciprocal role of the mesoderm in maintaining and determining the form of the ridge. These studies, noteworthy for their ingenuity and for the technical skill on which they depended, led Edgar to investigations of the emergence and fixation in the limb bud of the morphogenetic quality of "limbness," that is, the capacity to form a limb. He showed that this property is already present even before a definite limb bud forms; that it is a result of patterned cell-cell interactions; that it survives the disaggregation of limb mesoderm cells to reappear after their reaggregation; and that it persists until individual cells begin their overt differentiation into muscle, cartilage, and the like. The transition from a morphogenetic to a cytodifferentiative phase occurs gradually over several hours; it is preceded by the emergence in very small amounts of those end product molecules and enzyme activities that will subsequently characterize the full blown cytodifferentiative phase.

Since these contributions as well as their significance have already been reviewed in earlier biographical essays (Edds, 1972, *Developmental Biology*, **28**: 1; Saunders, 1971, *Developmental Biology*, **26**: 165), they will not be examined further here except to recall Edgar's preoccupation with the double thesis that, first, the emergence of form, especially at the organ level, is the central mystery of development, and its unravelling is the main goal of developmental biology; and, second, that obsession with any single analytical approach will always result in falling short of the goal. As he put it, "a complete understanding of the ontogeny of functional form will eventually relate the molecular, cellular, or supracellular phenomena responsible for the elaboration of form to the synthetic activities of the fully differentiated cells." In this uncompromising commitment to that central theme, he left a memorable heritage of basic contributions to embryology. In the warmth and generosity of his equal commitment to family, to friends, to students, to colleagues, and to the institutions he served, Edgar Zwilling left a large, indelible mark on hundreds of lives.

2. The Staff

EMBRYOLOGY

I. Consultants

Everett Anderson, Professor of Biology, University of Massachusetts

Anthony Clement, Professor of Biology, Emory University

Donald P. Costello, Professor of Zoology, University of North Carolina

II. Instructors

Malcolm S. Steinberg, Professor of Biology, Princeton University, in charge of course

Max Burger, Associate Professor of Biology, Princeton University

Ralph Hinegardner, Associate Professor of Biology, University of California, Santa Cruz

Hans Laufer, Associate Professor of Zoology, University of Connecticut

Eric Davidson, Associate Professor of Biology, California Institute of Technology

Gary Freeman, Assistant Professor of Biology, University of California, San Dieg

III. Special Lecturers

John Arnold, Associate Professor of Cytology, University of Hawaii
Raymond Rappaport, Professor of Biology, Union College
Paul B. Weisz, Professor of Biology, Brown University

IV. Laboratory Assistants

Anthony W. Shermoen, Wesleyan University
David M. Miyamoto, Duke University

V. Lectures

M. S. Steinberg	Introduction to the course
	Development of coelenterates
	Control processes in coelenterate development
J. P. Trinkaus	Analysis of teleost development (I)
	Analysis of teleost development (II)
R. Hinegardner	Echinoderm development: egg to pluteus
	Echinoderms: life cycle and experimental embryology
M. S. Steinberg	Morphogenetic phenomena in sponges
E. Anderson	Ultrastructure of oocytes
Raymond Rappaport	Cytokinesis I
	Cytokinesis II
H. Laufer	Hormonal control of differentiation
	Chromosomal puffing: its developmental significance
Robert Briggs	Genetic control of the egg cytoplasm
Ronald H. Reeder	Isolation and transcription of the genes for ribosomal RNAs
Yoshiaki Suzuki	Induction and sequencing of the mRNA for silk fibroin
Eric Davidson	Sequence homology studies with eukaryotic nucleic acids
Ralph Hinegardner	Evolution of cellular DNA content
Lajos Piko	Fertilization
Max Burger	Cell surface changes in neoplasia and during cytokinesis
Anthony Clement	Early development of spiralians
	Experimental analysis of spiralian development
John Arnold	Experimental studies on cephalopod development
Gary Freeman	Organization and early development of the ascidian egg
	The cellular basis of asexual reproduction in ascidians
Beth Burnside	The roles of microtubules and microfilaments in morphogenesis
M. S. Steinberg	Does differential cell adhesion govern self-assembly processes in morphogenesis? I. Behavioral evidence
Herbert M. Phillips	II. Precise formulation and direct physical testing of the differential adhesion hypothesis
	III. Application of the differential adhesion hypothesis to early amphibian morphogenesis
Antone Jacobson	Experiments on the control of organ determination
Johannes Holtfreter	Embryonic induction and morphogenetic fields
Paul B. Weisz	The significance of larvae
David S. Barkley	Pattern formation in aggregates of developing neural tissue
Viktor Hamburger	Neurogenesis and the origins of behavior

VI. Post Course Period

Thomas Roth	Physiological and structural evidence for protein transport into oocytes
Robert O. Becker	Stimulation of partial limb regeneration in mammals

PHYSIOLOGY

I. Consultants

Albert Szent-Györgyi, Director, The Institute of Muscle Research, Marine Biological Laboratory
W. D. McElroy, National Science Foundation
J. Woodland Hastings, Professor of Biology, Harvard University

II. Instructors

Andrew G. Szent-Györgyi, Professor of Biology, Brandeis University, in charge of course
Roderick K. Clayton, Professor of Biophysics, Cornell University
Lawrence Grossman, Professor of Biochemistry, Brandeis University
Hugh E. Huxley, Medical Research Council, Laboratory of Molecular Biology, Cambridge, England
Peter C. Newell, Department of Biochemistry, University of Oxford, Oxford, England
David A. Yphantis, Professor of Biology, University of Connecticut

III. Special Lecturers

Harlyn O. Halvorson, Professor of Bacteriology, University of Wisconsin
Shinya Inoue, Professor of Biology, University of Pennsylvania

IV. Staff Associates

Raymond E. Stephens, Department of Biology, Brandeis University
Annemarie Weber, Department of Biochemistry, St. Louis University
Richard J. Podolsky, Laboratory of Physical Biology, National Institute of Arthritis and Metabolic Diseases
Jacob Franke, Department of Biology, Brandeis University
Michael Johnson, Department of Biophysics, University of Connecticut
Walter F. Stafford, III, Department of Biophysics, University of Connecticut
Robert Haselkorn, Department of Biophysics, University of Chicago
William Lehman, Department of Biology, Brandeis University
Andrew Braun, Department of Biochemistry, Brandeis University

V. Research Assistants

Ruth Hoffman, Department of Biology, Brandeis University
Evelyn Wood, Department of Biophysics, Cornell University
Paul Sapin, Boston University
Gail Clinton, Department of Biology, University of California at La Jolla
Joan Newell, Department of Biochemistry, University of Oxford, Oxford, England

VI. Course Assistant

Mary van Holde, University of Oregon

VII. Lectures

Andrew G. Szent-Györgyi	Aspects of the chemistry of contraction
Annemarie Weber	Regulation of contraction
Andrew G. Szent-Györgyi	The proteins, and their assembly in molluscan muscles
David A. Yphantis	Physical biochemistry I
John G. Nichols	Specific connection and regeneration patterns of sensory and motor nerve cells of the leech nervous system
David A. Yphantis	Physical biochemistry II
	Physical biochemistry III
Lawrence Grossman	Mechanism of replication *in vivo* and *in vitro* I
	Mechanism of replication *in vivo* and *in vitro* II
Jeffries Wyman	Principles of linkage
	Illustrations in biological macromolecules
Lawrence Grossman	Enzymatic repair of DNA
Roderick K. Clayton	Photosynthesis I
	Photosynthesis II
Richard J. Podolsky	Control of contraction
Henry R. Mahler	Mitochondrial DNA and mitochondrial mutation
Edward Adelberg	Transfer replication of DNA in *E. coli*
Maurice S. Fox	On integration of DNA in bacterial transformation
Peter C. Newell	Developmental control in slime molds I
	Developmental control in slime molds II
Hugh E. Huxley	Structural aspects of muscle contraction I
	Structural aspects of muscle contraction II
Shinya Inoué	Biophysical analysis of mitosis in living cells I
	Biophysical analysis of mitosis in living cells II
R. E. Stephens	Biochemistry of microtubular systems I
	Biochemistry of microtubular systems II
Anthony C. H. Durham	How tobacco mosaic virus assembles
Michael F. Moody	Structure and contraction of the bacterial phage tail
Robert Haselkorn	The physiology of the bacteriophage T_4 development
Harlyn O. Halvorson	Meiosis in yeast
Lewis G. Tilney	Microtubules in development of cell forms
Laszlo Lorand	Enzyme catalyzed protein assemblies, fibrin
David Shemin	Enzymes in heme synthesis
Albert Szent-Györgyi	Water, motion and muscle
Seymour S. Cohen	The physiology, biochemistry and molecular biology of polyamines
Akira Kaji	On the mechanism of protein synthesis
Cyrus Levinthal	Symmetry in brains of little animals
Bernard D. Davis	The ribosome-polysome cycle
Jean-Pierre Changeux	Studies of cholinergic receptor protein in the electric eel
Jonathan B. Wittenberg	What does myoglobin do?
Salvador E. Luria	Colicins
Gustav V. R. Born	Adhesion of thrombocytes and leucocytes
Lawrence B. Cohen	Optical studies of action potentials
J. Woodland Hastings	Bioluminescence: endosymbiosis in the ponyfish and subunit functions in luciferase
Adolph I. Cohen	Rods and cones
Ruth Hubbard	Photochemistry of visual pigments
William Hagins	Studies on exitation in outer segments of rods
Ed Kravitz	Studies on synaptic chemistry in single nerve cells

David Hubel	Architecture and integration in the visual cortex of the brain
Nigel W. Dow	Neurophysiology of color vision

VIII. Special Lecture

Allan Weed	Comparative studies of the light chains of myosin

EXPERIMENTAL MARINE BOTANY

I. Consultants

Sterling B. Hendricks, National Academy of Sciences
Bessel Kok, Research Institute for Advanced Studies
Lawrence Gogorad, Harvard University

II. Instructors

Harold W. Siegelman, Brookhaven National Laboratory, in charge of course
Robert L. Guillard, Woods Hole Oceanographic Institution
Synnove Liaaen Jensen, Norwegian Institute of Technology
Arne Jensen, Norwegian Institute of Technology
Frank A. Loewus, State University of New York at Buffalo
Anthony G. San Pietro, Indiana University
Jerome A. Schiff, Brandeis University
Michael J. Wynne, University of Texas

III. Special Lecturers

Joseph Mascarenhas, State University of New York at Albany
N. Keith Boardman, C.S.I.R.O., Australia
R. P. Levine, Harvard University
Shimon Klein, Hebrew University, Israel
Donald J. Plocke, Boston College
Mordhay Avron, Weizmann Institute, Israel
Otto Kandler, University of Munich, Germany
W. W. Youngblood, Woods Hole Oceanographic Institution and Florida Technological University
Bjorn Larson, Norwegian Institute for Seaweed Research, Norway
Norman Krinsky, Tufts University School of Medicine
Arthur Stern, University of Massachusetts, Amherst

IV. Research Associates

Martin Gibbs, Brandeis University
Carl A. Price, Rutgers University

V. Research Assistants

George Wagner, State University of New York at Buffalo
Richard Wetherbee, University of Michigan

VI. Lectures

H. W. Siegelman	Photobiologically active plant chromoproteins
Arne Jensen	Chemistry of the algal classes
Synnove Liaaen-Jensen	Carotenoids–general aspects
	Comparative biochemistry of marine carotenoids

Arne Jensen	Perspectives in algal chemistry
	Utilization of seaweeds
Robert L. Guillard	Cultures as tools for the study of phytoplankton ecology
	Physiological races of marine planktonic diatoms
	Limitation of phytoplankton growth by low nutrient levels
Michael J. Wynne	Introduction to the benthic marine algae: chlorophyta
Jerome A. Schiff	Sulfate metabolism in algae
Michael J. Wynne	Introduction to the benthic marine algae: rhodophyta and phaeophyta
Joseph Mascarenhas	Intracellular structures and their movements in plant cells
Michael J. Wynne	Vertical zonation and distribution patterns along the atlantic coast
N. Keith Boardman	The photosynthetic process
Jerome A. Schiff	Plastid structure and evolution
	Plastid development and inheritance
R. P. Levine	Genetics of photosynthesis and the chloroplast
Jerome A. Schiff	Evolution of photosynthetic pigment systems
Shimon Klein	Comparative aspects of chloroplast development in *Euglena* and higher plants
Anthony San Pietro	Photosynthetic electron transport and photophosphorylation. I. Introduction
	II. Components of the system
	III. Current view
Donald J. Plocke	Altered ribosomes in zinc- and iron-deficient microorganisms
Mordhay Avron	Electron transport and carbon metabolism in photosynthesis
Otto Kandler	Physiology and biosynthesis of Hamamelose
Martin Gibbs	Photorespiration
W. W. Youngblood	Hydrocarbons in algae
F. Loewus	Carbohydrate interconversions involving inositol
Carl A. Price	Zonal centrifugation and particle separation
F. Loewus	A functional role for carbohydrate exudate of plants
Bjorn Larsen	Biosynthesis of alginic acid
Norman Krinsky	Protective function of carotenoid pigments
Arthur Stern	Photophosphorylation and ATPase activity in mature and developing chloroplasts

EXPERIMENTAL INVERTEBRATE ZOOLOGY

I. Consultants

Frank A. Brown, Jr., Professor of Zoology, Northwestern University
C. Ladd Prosser, Professor of Physiology, University of Illinois
Clark P. Read, Professor of Biology, Rice University
Alfred C. Redfield, Woods Hole Oceanographic Institution
W. D. Russell-Hunter, Professor of Zoology, Syracuse University

II. Instructors

James F. Case, Professor of Biology, University of California, Santa Barbara, in charge of course
Garth Chapman, University College, London
Alan Gelperin, Assistant Professor of Biology, Princeton University

David C. Grant, Assistant Professor of Biology, Davidson College
Michael J. Greenberg, Associate Professor, Florida State University
Joseph B. Jennings, Department of Zoology, University of Leeds
Charlotte P. Mangum, Associate Professor of Biology, College of William and Mary
James G. Morin, Assistant Professor of Zoology, University of California, Los Angeles
Dorothy M. Skinner, Biology Division, Oak Ridge National Laboratory

III. Special Lecturers

Thomas J. M. Schopf, University of Chicago
G. Reynolds, Princeton University
William Stewart, University of California, Santa Barbara
R. L. Pardy, University of California, Los Angeles
Demorest Davenport, University of California, Santa Barbara
John H. Todd, Woods Hole Oceanographic Institution
Kenneth D. Roeder, Tufts University
Fotis Kafatos, Harvard University
Audrey E. V. Haschemeyer, Hunter College
Jeffrey M. Camhi, Cornell University

IV. Assistants

Arnold G. Eversole, Syracuse University
Eve C. Haberfield, University of Rhode Island
George A. Kahler, III, Rice University

V. Lectures

J. Case	Introduction Invertebrate phylogeny
J. Morin	Porifera and Cnidaria Cnidaria and ctenophores
G. Chapman	Flatworms, nemerteans, aschelminthes
T. Schopf	Ectoprocts
C. Mangum	Annelida
M. Greenberg	Mollusca I Mollusca II
D. Skinner	Arthropoda I Arthropoda II
G. Chapman	Echinoderms
J. Case	Protochordates
Demorest Davenport	Computerization studies of the orientation of microscopic organisms
John H. Todd	An ethology of stress environments
C. Mangum	Respiration. I. Exchange Respiration. II. Transport
M. Greenberg	Patterns of circulation among the invertebrates Hearts and visceral muscle: the way to a clam's heart is through its rectum Some aspects of comparative muscle physiology
C. Mangum	Temperature adaptation
A. Gelperin	Regulation of feeding

J. Jennings	Alimentary systems
	Digestive physiology—with particular reference to acoelomates I.
	Digestive physiology—with particular reference to acoelomates II.
Fotis Kafatos	Hormone-initiated cellular differentiation in insects
J. Morin	Primitive nervous systems
A. Gelperin	Complex behavior in simple neural systems
	Endogenous activity and rhythmic behaviors
	Command and executive neurons
J. Case	Invertebrate photoreception
Kenneth D. Roeder	Palps and pilifers
J. Case	Invertebrate chemoreceptors
G. Reynolds	Methods in the investigation of bioluminescence
J. Morin	Bioluminescence in the lower metazoa
William Stewart	Factors influencing larval settlement
D. Skinner	Growth and molting I
	Growth and molting II
J. Case	Bioluminescence in higher metazoa
Audrey E. V. Haschemeyer	Studies on the mechanism of temperature acclimation of marine organisms
Jeffrey M. Camhi	Diverse functions of the locust flight motor
R. L. Pardy	Algal symbiosis in coelenterates

MARINE ECOLOGY

I. Consultants

Melbourne R. Carriker, Marine Biological Laboratory
Howard L. Sanders, Woods Hole Oceanographic Institution
Lawrence B. Slobodkin, State University of New York at Stony Brook
Roger Y. Stanier, University of California at Berkeley

II. Instructors

Holger W. Jannasch, Senior Scientist, Woods Hole Oceanographic Institution, in charge of course
Richard W. Castenholz, Professor of Botany, University of Oregon at Eugene
Ralph Mitchell, Professor of Applied Microbiology, Harvard University
Sumner Richman, Professor of Biology, Lawrence University
Donald C. Rhoads, Associate Professor of Geology, Yale University
Edward O. Wilson, Professor of Zoology, Harvard University

III. Special Lecturers

Jelle Atema, Woods Hole Oceanographic Institution
Ercole Canale-Parola, University of Massachusetts at Amherst
Frederick J. Grassle, Woods Hole Oceanographic Institution
J. Woodland Hastings, Harvard University
Galen E. Jones, University of New Hampshire
Edward R. Leadbetter, Amherst College
Kenneth Nealson, Harvard University
John D. Palmer, New York University

Sydney C. Rittenberg, University of California at Los Angeles
Howard L. Sanders, Woods Hole Oceanographic Institution
Lawrence B. Slobodkin, State University of New York at Stony Brook
John M. Teal, Woods Hole Oceanographic Institution
John Todd, Woods Hole Oceanographic Institution
Wolf Vishniac, University of Rochester
Ralph S. Wolfe, University of Illinois at Urbana

IV. Laboratory Assistants

Herman F. Bosch, Johns Hopkins University
Denis Cunningham, Yale University

V. Lectures

H. W. Jannasch	Introduction to microbial ecology
	Marine microbiology I and II
	Continuous culture in microbial ecology
	Theory and practice of continuous culture techniques
	Microbiology of the Black Sea
	Ecology of photosynthetic bacteria
E. R. Leadbetter	How organisms make a living
	Methods of microbial ecology
S. C. Rittenberg	Microbiology of marine sediments
	The microbial sulfur cycle
R. S. Wolfe	Anaerobic transformations in the cycle of matter
	Anaerobic microörganisms in marine sediments
R. Mitchell	Intermicrobial predation
	Ecological aspects of bacterial chemotaxis
	Attachment of bacteria to surfaces
	Unusual iron and manganese bacteria
	Marine fouling
	Bacterial aggregation
	Microbial approaches to water pollution control
J. W. Hastings	Symbiosis of luminescent bacteria
K. Nealson	Problems in the classification of marine bacteria
R. W. Castenholz	Photosynthesis and productivity in the sea
	Light in natural waters; effects on photosynthesis and growth
	Inorganic nutrients as factors limiting the growth of phytoplankton
	Organic factors limiting growth of phytoplankton
	Intertidal zonation of macro-algae
	The ecology of hot springs I and II
E. Canale Parola	Biology of Spirochaetes
	Intermediary metabolism of Spirochaetes
S. Richman	Food chains and ecological efficiencies
	Energetics of single species
	Quantitative methods for measuring the feeding of planktonic Crustacea
	Assimilation of food by zooplankton
	Studies on the feeding behavior of copepods I and II
J. M. Teal	Salt marsh ecology
	Experimental work on the Sippewissett salt marsh
H. F. Bosch	The marine Cladocera

D. C. Rhoads	Structure and dynamics of benthic invertebrate assemblages
	Taxonomy of Buzzards Bay benthic communities
	The trophic structure of benthic communities I and II
	Sedimentology for ecologists
	Evolutionary and ecologic significance of oxygen-poor environments
	The evolution of benthic communities, pre-Cambrian to recent
F. J. Grassle	Benthic sampling
	Genetic variability of benthic communities
H. L. Sanders	Biological effect of an oil spill
E. O. Wilson	Principles of biogeography I and II
	Principles of speciation I and II
	Chemical communication I and II
	Principles of Sociobiology
J. Atema	Chemical communication III
J. Todd	An ethology of stress environment
G. E. Jones	The fate of freshwater bacteria in the sea
	The significance of heavy metals to marine bacteria
W. Vishniac	Productivity and exploitation of the ocean
	Planetary and biological evolution
L. B. Slobodkin	Ecology, a science, a movement, and a crisis

NEUROBIOLOGY

I. Instructors

Michael V. L. Bennett, Professor of Anatomy, Albert Einstein College of Medicine, Yeshiva University, co-director of course

John E. Dowling, Associate Professor of Ophthalmology and Biophysics, Johns Hopkins University School of Medicine, co-director of course

Rodolfo R. Llinas, Associate Member, Education and Research, Foundation of the American Medical Association

George Pappas, Professor of Anatomy, Albert Einstein College of Medicine, Yeshiva University

Felix Strumwasser, Professor in the Division of Biology, California Institute of Technology

Victor Whittaker, Sir William Dunn Reader in Biochemistry, Cambridge University

II. Special Lecturers

Stephen G. Waxman, Postdoctoral fellow, Albert Einstein College of Medicine

George Katz, Assistant Professor, Columbia University

A. L. F. Gorman, Research Physiologist, National Institute of Mental Health

Maurizio Mirolli, St. Elizabeth Hospital, Washington D. C.

R. L. Chapell, Assistant Professor, Hunter College

John Lisman, Massachusetts Institute of Technology

Charles Nicholson, Assistant Professor, University of Iowa

III. Lectures

George D. Pappas	Nerve cells and the fine structure of membranes
	Structure and function of synapses
Stephen G. Waxman	Myelin and nodes of Ranvier

M. V. L. Bennett	Central dogma of neuro-physiology. I. Impulses Central dogma of neuro-physiology. II. Synapses
George Katz	Introduction of electrophysiological measurements
A. L. F. Gorman	Membrane theory: properties of nerve membrane Membrane potential of nerve and glial cells and diffusion through the extracellular space
Maurizio Mirolli	Geometrical factors determining the electrotonic properties of nerve cells
A. L. F. Gorman	Metabolism of nerve cells: Na-K exchange pump and electrogenic effects Specialized regions of nerve cells: Synaptic and sensory membrane
J. E. Dowling	Introduction to the visual system: Anatomy, chemistry, receptor potentials The lateral eye of *Limulus*
R. L. Chappell	Synaptic activation by receptor slow potentials
J. E. Dowling	The processing of visual information
A. L. F. Gorman	Comparative physiology of hyperpolarizing photoreceptor potentials
M. V. L. Bennett	Iontophoretic application of drugs and micropharmacology Electric organs: comparative physiology and experimental utility
A. B. Steinbach	Transmission at synapses of electroreceptors
M. V. L. Bennett	Functions of electrotonic synapses Interpretation of intracellular recordings in the CNS
Rodolfo Llinas	Cerebellar electrophysiology as a model for the analysis of central neuronal networks
John Lisman	*Limulus* photoreceptors: The role of Ca^{++} in light-adaptation
Charles Nicholson	Analysis of field potentials evoked by populations of spatially oriented central neurons
Rodolfo Llinas	Dynamic properties of the responses of cerebrellar neuronal circuits to natural stimuli

SYSTEMATICS-ECOLOGY PROGRAM

The Staff

Director: Melbourne R. Carriker

Resident Systematist (Zoology): Arthur G. Humes

Acting Resident Systematist (Botany): Robert T. Wilce

Resident Ecologist: Ivan Valiela

Resident Environmental Physiologist: William C. Stewart

Curator (Zoology): Johanna M. Reinhart

Curator (Botany, part time): Wesley N. Tiffney

Postdoctoral Fellows and Research Associates: James Fiore, Raymond P. Markel, Lawrence R. McCloskey, Allan D. Michael, Leland W. Pollock, William J. Woelkerling

Graduate Research Trainees: John Aldrich, Charlene D'Avanzo, Bruce W. Found, Walter Hatch, Stewart Jacobson, Warren Kaplan, Charles Krebs, Joe Wright, Roy M. Yarnell

Visiting Investigators: Marie B. Abbott, Louise Bush, Patricia L. Dudley, Joel S. O'Connor, Philip Person, Harold H. Plough, Wesley N. Tiffney, Ruth D. Turner, William C. Summers, William J. Woelkerling, David K. Young

Collaborators: David Downing, Virginia Peters, James Schaadt
Visitors: Donald Bourne, Katharine D. Hobson
Consultants: William Randolph Taylor, Ruth D. Turner, Robert T. Wilce
Administrative Assistant: Constance A. Brackett
Program Secretary: Eva S. Montiero
Technical Field Assistant: Peter J. Oldham
Scientific Illustrators: Ruth von Arx, Susan P. Heller
Captains, R/V A. E. Verrill: Peter Graham, Francis J. Doohan
Mates: Francis J. Doohan, Stephen K. Young
Boatswain: William Plettner
Research Assistants: Ann Cody, Gene Crouch, Theodore J. Grant (Bibliographer), Dudley L. Greeley, Patricia Hughes, Tracy McLellan, John J. McMahon, Nancy McNelly, Margaret A. Mills (Biotic Census Project Ass't), Helen Ortins, Carol Parmenter, Paul V. Ruhlman, Greg Ruppert, Warren Sass (computer operator), Carol Q. Schwamb, Martha Swartz, Eric Teal, Richard J. Traverse, Dirk Vanzandt, Mary Lee P. Wilson, Richard Wynne
Participants: Nell Backus, Shirley Holbrook

SEP Seminars (Winter Included)

Roy M. Yarnell	What I am doing in the salt marsh
Poul Heegaard	Ovarial structures in the penaeids and larval stages and growth in the decapods
Harold H. Plough	Distribution changes in ascidian species on the continental shelf: evolution in fifty years
Eldon Ball	Electrical correlates in behavior in the solitary hydroid, *Corymorpha palma*
Edward Carpenter	Distribution of diatoms on pelagic *Sargassum* and utilization of urea by some marine phytoplankters
Joel S. O'Connor	Benthic invertebrates of Moriches Bay, Long Island
William C. Stewart	A study of the nature of the attractant in the *Ophiodromus pugettensis-Patiria lineata* commensal association
Robert Blumberg	Legal problems relating to the oceanic environment in Massachusetts
Donald J. Zinn	Exploitation of the Alaskan Tundra
Paul E. Hargraves	Observations on phytoplankton of Narragansett Bay, Rhode Island
John C. Hathaway	Composition and movement of fine grained sediments along the Atlantic Coast
Lawrence R. McCloskey	Tektite II: impressions, decompressions, and digressions
Herbert W. Graham	What happened to BCF?
Dennis Polis and Don Maurer	Planning for the baseline study of the Delaware Bay
George P. Fulton	Trends in microvascular research (illustrated by cinephotomicroscopy)
James D. Lazell	Herpetology of Cape Cod and the Islands: problems of distribution
Henry Campbell	The role of the Coast Guard in water pollution
Randall B. Fairbanks	An assessment of the effects of power generation upon the Cape Cod Canal sport fishery
Brian D. Bornhold and John D. Milliman	Phylogenetic and environmental influences upon the composition of serpulid (Polychaeta) tubes
Willard D. Hartman	Some living fossils among the sponges
Marvin C. Meyer	Taxonomy of marine leeches—in retrospect and prospect

Marie B. Abbott	Bryozoan populations of Block Island Sound
John H. Dearborn	Ecological studies of polar echinoderms
Richard C. Kugler	Whaling under sail: some aspects of the American experience
Arthur G. Humes	Copepods associated with marine invertebrates
Paul Tessier	Biological adaptations of marine sand microfauna; and *Halamohydra* (film)
Edward Gilfillan	Physiological changes occurring during the invasion of the coastal environment by an oceanic species of zooplankton, *Euphausia pacifica* Hansen
Ellsworth Wheeler	On the ecology of carnivorous Copepoda
Paul Godfrey	Ecological implications of oceanic overwash: dunes, dikes, and engineers
Bruce Coull	Shallow water meiobenthos of the Bermuda platform
Allan D. Michael	Environmental factors and species distribution in Cape Cod Bay
Henry M. Reiswig	Comparative physiology of field populations of Jamaican Demospongiae
Kenneth Turgeon	The effects of cornstarch and dextrose supplements on oysters
John Field	Cluster analysis: a tool for studying marine benthic communities
H. P. Jeffries	Phytoplankton-zooplankton relationships: aspects of a biochemical enigma
Richard L. Miller	Chemotaxis of coelenterate sperm
H. Burr Steinbach	The Woods Hole Oceanographic Institution educational program
Berton Roffman	Algal symbiosis
Roland L. Wigley	Biology of the northern shrimp
Richard Hennemuth	Exploitation and management of fishery resources of the northwest Atlantic
James Parmentier	Analysis of glutamic acid receptor sites in gastropod neurons
Oscar Liu	Effects of chlorination on viruses in water
Frank Belamarich	Aggregation of amoebocytes in *Limulus*
Robert Livingstone	Haddock spawning and maturity studies—past and present
John Lee	The microbiology of 1 cc of a salt marsh epiphytic community
Robert K. Selander	Systematic applications of the allozyme technique
R. W. Castenholz	Species interaction in a hot spring
William Cooper	Recent views on systems analysis of ecosystems
Sumner Richman	Selective feeding behavior of Woods Hole copepods

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Kropp, Donna L., Syracuse University
Langer, George Scott, Lawrence University
Lanni, Carmine, Herbert Lehman College of City University of New York
Lee, David, Ottawa University
Leetmaa, Bonnie L., University of Massachusetts
Lehman, William J., Brandeis University
Leith, Ardean, University of Rochester
Lipson, Robert A., Columbia University
Lisman, John Massachusetts Institute of Technology
Loveday, Kenneth S., Massachusetts Institute of Technology
Luzzati, Anne, Orsay, France
MacKay, Alexander R., Princeton University
Mazal, Dennis, Rutgers University
McCall, Peter, Yale University
Milch, James Roger, Princeton University
Mineconzo, Gary A., Union College
Miyamoto, David Mark, Duke University
Mobberly, Deborah Kay, Tulane University
Moore, Marilyn R., University of Connecticut Health Center
Mooseker, Mark S., University of Pennsylvania
Moran, Nava, The Hebrew University
Natalini, John J., Northwestern University
Noonan, Kenneth D., Princeton University
O'Rand, Angela M., Temple University
O'Rand, Michael G., Temple University
Orsini, Roger A., University of Connecticut
Osman, Richard W., University of Chicago
Parmentier, James L., University of California, Santa Barbara
Patton, Alice, University of Texas at Austin
Pencek, Terrence L., Illinois Institute of Technology
Perrelet, Alain, The Rockefeller University
Persell, Roger, Hunter College
Pilapil, C., University of Montreal
Preddie, Enrique C., University of Montreal
Reusch, Victor M., Princeton University
Roberts, James T., Massachusetts General Hospital
Robertson, Mrs. C. W., American Museum of Natural History
Rosen, Jeffrey B., Case Western Reserve University
Rowland, Andy, University of Pennsylvania
Saccone, James M., University of Connecticut Medical School
Sapin, Paul, Cleveland, Ohio
Schnur, Thomas, Oakland University
Scotto, Joseph M., Institute for Basic Research, Staten Island
Shapiro, Edward J., State University of New York at Buffalo
Shapiro, Robert Alan, Oakland University
Shermoen, Antony W., Wesleyan University
Shick, J. Malcolm, College of William and Mary
Shiroky, Dorothy V., Johns Hopkins University
Snyder, David A., Brown University and University of Southern California Medical School
Snyder, Thomas P., Juniata College

Sperling, Linda, Harvard University
Sternfeld, John, University of California, San Diego
Stevens, E. D., University of Hawaii
Stiller, Ronald A., Boston University
Stillings, Susan N., Oberlin College
Stillings, Wayne, Oberlin College
Strickholm, Steve, Indiana University
Sulanowski, Jacek, Wayne State University
Sussman, Paul, Columbia University
Swanson, Ruth Ann, Wayne State University
Szamier, R. Bruce, Albert Einstein College of Medicine
Szönyi, Eszter I., Boston University School of Medicine
Szumilas, Diana, Wayne State University
Taylor, Grantley W., New York University School of Medicine
Tsang, Monica Lik-Shing, Brandeis University
Tsiringas, Harissios F., State University of New York at Buffalo
Tucker, Gail Susan, University of Kansas
Turansky, David G., Columbia University
Turetsky, Oxana, Brooklyn College
Uppal, J. S., University of Montreal
Van Holde, Mary Annette, University of Oregon
Wagner, George J., State University of New York at Buffalo
Weck, Steven D., New York University Medical School
Weiss, Jonathan, Wesleyan University
Wetherbee, Richard, University of Michigan
Wexler, Andrew, Dartmouth College
Wintemute, Garen John, Yale University
Woodard, Brett H., University of Pittsburgh
Youngdahl, Pamela E., University of Oregon
Yulo, Teresa S., University of Rochester
Zakevicius, Jane M., New York University Medical Center
Zdunski, H. Duleine, Case Western Reserve University

Library Readers, 1971

Allen, Garland E., Assistant Professor of Biology, Washington University
Allen, Robert Day, Professor and Chairman, Department of Biological Sciences, State University of New York at Albany
Anderson, Rubert S., Independent Library Reader, Marine Biological Laboratory
Ball, Eric G. Professor Emeritus of Biological Chemistry, Harvard University
Bendet, Irwin J., Professor of Biophysics, University of Pittsburgh
Berlin, Richard D., Associate Professor of Physiology, Harvard Medical School
Berne, Robert M., Professor and Chairman, Department of Physiology, University of Virginia School of Medicine
Boettiger, Edward G., Professor of Physiology, University of Connecticut
Bridgman, Anna Josephine, Professor and Chairman, Department of Biology, Agnes Scott College
Buck, John, Chief, Laboratory of Physical Biology, National Institutes of Health
Carlson, Francis D., Professor of Biophysics, Johns Hopkins University
Cassidy, Fr. Joseph D., Assistant Professor of Biology, University of Notre Dame
Child, Frank M., Associate Professor of Biology, Trinity College
Clark, Arnold M., Professor of Biological Sciences, University of Delaware
Cohen, Seymour S., Professor and Chairman, Department of Therapeutic Research, University of Pennsylvania, School of Medicine
Copeland, Donald Eugene, Professor of Biology, Tulane University
Couch, Ernest F., Assistant Professor of Biology, Texas Christian University
Crowell, Sears, Professor, Department of Zoology, Indiana University
Davis, Bernard D., Professor of Bacterial Physiology, Harvard Medical School
Dudley, Patricia L., Associate Professor of Biology, Barnard College

EBNER, FORD F., Associate Professor, Brown University
EDER, HOWARD A., Professor of Medicine, Albert Einstein College of Medicine
EISEN, HERMAN N., Professor of Microbiology, Washington University
GABRIEL, MORDECAI L., Professor and Chairman, Biology Department, Brooklyn College
GELFANT, GRACIELA C. CANDELAS, Professor, Department of Biology, University of Puerto Rico at Rio Piedros
GERMAN, JAMES L., Investigator, and Director of the Laboratory of Human Genetics, The New York Blood Center and Associate Professor, Cornell University Medical College
GINSBERG, HAROLD S., Professor and Chairman, Department of Microbiology, University of Pennsylvania
GREEN, JAMES W., Professor of Physiology, Rutgers University
GROSS, PAUL R., Professor of Biology, Massachusetts Institute of Technology
GUSSIN, ARNOLD E. S., Assistant Professor, Smith College
HASTINGS, J. Woodland, Professor, Biological Laboratories, Harvard University
HILLMAN, NINA W., Research Associate, Temple University
HILLMAN, RALPH, Professor, Temple University
HOLTZER, HOWARD, Professor, University of Pennsylvania
JOHNSON, EDWARD A., Graduate Student, University of New Hampshire
KEMPTON, RUDOLF T., Professor Emeritus of Biology, Vassar College
KEOSIAN, JOHN, Professor Emeritus, Rutgers University, The State University of New Jersey
KIRSCHENBAUM, DONALD M., Associate Professor of Biochemistry, College of Medicine Downstate Medical Center
KRASSNER, STUART M., Vice Chairman Department of Developmental and Cell Biology and Associate Professor, University of California, Irvine
KRAVITZ, EDWARD A., Professor of Neurobiology, Harvard Medical School
LEE, HAROLD H., Assistant Professor, University of Toledo
LEVY, ARTHUR L., Chief of Clinical Chemistry, St. Vincent's Hospital and Medical Center of New York
LURIA, SALVADOR E., Institute Professor of Biology, Massachusetts Institute of Technology
MARSLAND, DOUGLAS, Research Professor Emeritus, New York University
MAUTNER, HENRY G., Chairman, Department of Biochemistry and Pharmacology, Tufts University School of Medicine
MIZELL, MERLE, Professor of Biology, Tulane University
MORRELL, FRANK, Professor of Neurology and Psychiatry, New York Medical College
NASATIR, MAIMON, Professor of Biology, University of Toledo
PERSON, PHILIP, Chief, Special Research Laboratory, Veterans Administration Hospital, Brooklyn
PIKE, EILEEN H., Associate Professor of Parasitology, New York Medical College
PLOUGH, HAROLD H., Professor of Biology Emeritus, Amherst College
ROTH, JAY S., Professor of Biochemistry, University of Connecticut
ROTH, L. EVANS, Professor and Director, Division of Biology, Kansas State University
ROTH, OWEN H., Head, Department of Biology, St. Vincent College
ROWLAND, LEWIS P., Professor and Chairman, Department of Neurology, University of Pennsylvania
RUBINOW, S. I., Professor of Biomathematics, Cornell University Medical College
RYBICKA, KRYSTYNA, Research Associate, Rice University
SCHLESINGER, R. WALTER, Professor of Chemistry, Rutgers Medical Center
SCOTT, ALAN, Professor of Biology, Colby College
SHEMIN, DAVID, Professor of Biochemistry, Northwestern University
SILUNAS, KESTAS E., Associate Professor of Sociology, Pennsylvania State University
SMELSER, GEORGE K., Professor of Anatomy, Columbia University College of Physicians and Surgeons
SONNENBLICK, B. P., Professor of Zoology, Rutgers University
SONNENBLICK, EDMUND H., Associate Professor of Medicine, Harvard Medical School
STETTEN, DEWITT, JR., Director, NIGMS, National Institutes of Health
STETTEN, MARJORIE R., Chemist, NIAMD, National Institutes of Health
STRAUSS, ELLIOTT W., Associate Professor of Medical Sciences, Brown University
STRITTMATTER, PHILIPP, Professor of Biochemistry, University of Connecticut Health Center
TEREBEY, NICHOLAS, Lecturer, Department of Biological Structure, University of Washington

WAINIO, WALTER, Professor and Chairman, Department of Biochemistry, Rutgers-The State University of New Jersey
WEISS, LEON, Professor of Anatomy, Johns Hopkins Medical School
WHEELER, GEORGE E., Associate Professor of Biology, Brooklyn College
WICHTERMAN, RALPH, Professor of Biology, Temple University
WILSON, THOMAS HASTINGS, Professor of Physiology, Harvard Medical School
WITTENBERG, JONATHAN B., Professor of Physiology, Albert Einstein College of Medicine
YNTEMA, CHESTER L., Professor of Anatomy, State University of New York, Upstate Medical Center
ZEIDENBERG, PHILLIP, Instructor in Psychiatry, Columbia University, College of Physicians and Surgeons

Students, 1971

All students listed completed the formal course program. Asterisk indicates students completing post-course research program.

ECOLOGY

AVISE, JOHN C., University of Texas, Austin
*BALDWIN, RALPH W., Clark University
*CANNON, JOHN C., Williams College
CONNELL, MARY U., Kent State University
*EARLANDSON, RALPH P., University of Chicago
EIRIKSDOTTIR, GUDNY, Vassar College
GAVIS, JEROME, Johns Hopkins University
*JACOBS, NORMAN M., University of Pennsylvania
MESCHER, MATTHEW F., Harvard University
*MUNDY, PHILLIP R., University of Alabama
*MOUSALLI, ELIE, American University, Beirut
SHULENBERGER, ERIC, University of Kansas
*SZEKELY, DANIEL R., State University of New York at Stony Brook
THORSON, STUART H., University of Washington
*WAITE, THOMAS D., Harvard University
*WIDEGREN, ELISABETH J., Mississippi State College for Women
*WILSON, DAVID S., University of Rochester
*WORLEY, ANN C., Yale University

EMBRYOLOGY

*ALBERTINI, DAVID F., University of Massachusetts, Amherst
*BEEBE, DAVID C., University of Virginia
BENECCHI, JOHN L., Marquette University
*BRANDRIFF, BRIGITTE F., University of California, Santa Cruz
*CALVET, JAMES P., University of Connecticut
*CLARK, STEPHEN H., Wesleyan University
*COLBERT, DONALD A., Brown University
*EVANS, LEONARD E., Michigan State University
*GALLER, LYNNE, University of Chicago
*HEUSER, MARSHA, Georgetown University
*JOHNSON, LINDA M., Harvard University
*KERN, CLIFFORD H., III, Indiana University
KNAACK, LORETTA J., Rice University
*KORENBERG, JULIE R., University of Wisconsin
*KORNFELD, STEPHEN J., Union College
LEE, FRANK G., Oberlin College
*LEWIN, DAVID I., Yale University
MACMORRIS, MARGARET A., California Institute of Technology
MILLER, ELIZABETH T., University of Illinois
NEWMAN, GERALDINE R. GIOSA, Medical College of Pennsylvania
*PARENT, JAMES B., University of Virginia

*Rosenberg, Paul A., Albert Einstein College of Medicine
*Singley, Cart T., University of Hawaii
Sweadner, Kathleen, University of California, Santa Barbara
*Tanaka, Karen J., University of Minnesota
Warren, Audrey J., Princeton University
Weinbaum, George, Albert Einstein Medical Center
Woodward, John B., III, University of Colorado

EXPERIMENTAL BOTANY

*Aghajanian, John G., Long Island University
*Beck, Timothy A., University of California, Los Angeles
Bedigian, Dorothea, University of Vermont
Checkley, David M., Jr., Scripps Institution of Oceanography
*Clark, Robert L., University of Cincinnati
Cohen, Dan, Brandeis University
Davidson, Jeffrey N., Indiana University
*Derr, Janice A., Oberlin College
*DeVitis, Janet, Duke University
*Fetscher, Charles T., Hamilton College
*Fineman, Elliott L., Oberlin College
*Gauss, Verena, Mt. Holyoke College
*Goldstein, Marjorie F., Rutgers University
*King, Dan O., Jr., Indiana University
Klein, Nancy C., University of Virginia
Kleyn, John G., University of Puget Sound
McConnell, Maureen, University of Windsor
Ogus, Judith R., Bennington College
Peavey, Dwight G., Brandeis University
Simon, Jack H., State University of New York at Stony Brook
Singer, Ellen M., University of Massachusetts
Walker, Frederick J., University of California, San Diego

PHYSIOLOGY

*Abrahams, Susan Jane, Columbia University
*Biroc, Sandra Lyn, Johns Hopkins University
*Blum, Dr. Haywood, Drexel University
*Buzash, Elizabeth A., University of Connecticut
*Clay, John R., University of Rochester
Colton, Carol A., Rutgers Medical School
*Cooper, Jon C., University of Wisconsin
*Current, Steven P., University of Chicago
*Domanik, Richard A., Northwestern University
*Eaton, Barbra L., University of Pennsylvania
*Evans, Frederick E., State University of New York at Albany
*Gordon, Charles R., Massachusetts Institute of Technology
*Gulati, Jagdish, Pennsylvania Hospital
Huszar, Gabor D., M.D., Boston Biomedical Research Institute
Krantz, Allen, State University of New York at Stony Brook
*Linney, Elwood, University of California, San Diego
McElroy, James D., University of California, San Diego
*Meyers, Judy A., University of Pittsburgh
*Miller, Michael R., Milton S. Hershey Medical Center
*Pastuszyn, Andrzej, University of New Mexico
*Pearson, Terry W., University of California, Davis
*Popot, Jean-Luc, Institut Pasteur, Paris

Reichert, Thomas A., Carnegie-Mellon University
*Reid, Lola C. McAdams, University of North Carolina, Chapel Hill
*Sacevich, Eugene G., Johns Hopkins University
*Salmon, Edward D., University of Pennsylvania
*Siegal, Michael S., Columbia University, College of Physicians and Surgeons
*Simpson, Peter A., Brandeis University
*Thomas, Joseph M., Jr., Michigan State University
*Van Sambeek, Jerome W., Washington University, St. Louis
*Wallace, Douglas C., Yale University
*Warner, Cynthia K., University of Tennessee
Welch, George R., University of Tennessee

INVERTEBRATE ZOOLOGY

*Ang, Estrella Z., University of Pittsburgh
Atkinson, John M., University of Hawaii
Bates, Robert J., Yale University
Clark, Alvin J., University of California, Berkeley
*Cornell, John C., University of California, Berkeley
Coutchie, Pamela A., University of California, Davis
Diefenbach, Carlos O. da C., State University of New York at Buffalo
*Dougherty, James J., III, Rice University
Dohrmann, John D., National Marine Fisheries Service Groundfish Biology
Dudek, Francis E., University of California, Irvine
*Ellison, Anthony M., Yale University
Evans, Stephen J., University of California, Riverside
Eyman, Karen A., Kent State University
*Fenner, Douglas H., Reed College
Hill, Margaret C., University of Kansas
Huff, Annie L., Fort Valley State College
*Itaya, Stephen K., University of Tennessee
Johnson, Gary L., San Fernando Valley State College
*Knudsen, Eric I., University of California, Santa Barbara
LeFlore, William, Atlanta University
Marzouk, Joseph B., Princeton University
Miller, James R., Pennsylvania State University
Owen, Paul H., Jr., State University of New York at Syracuse
*Palmer, Lucy B., Smith College
Sammarco, Paul W., Syracuse University
*Shapiro, Eli, Yale University
*Torres, Joseph J., College of William and Mary
Wen, George Walter Sun, Harvard University
Westrom, Wendy K., Douglass College
Wilson, Maxine L., Emory University
*Winquist, Raymond J., University of California, Santa Barbara

NEUROBIOLOGY

Clusin, William T., Albert Einstein College of Medicine
Hochstein, Shaul Stephen, University of Jerusalem, Israel
Joyner, Ronald W., Duke University
Moran, David T., University of Colorado
Russell, John M., University of Utah
Sanes, Joshua R., Harvard University
Tweedle, Charles D., Yale University
Zaret, Wendy N., New York University Medical School

FRONTIERS IN RESEARCH AND TEACHING

Anekwe, Gregory, Tuskegee Institute
Ferguson, Thomas, Delaware State College
Huff, Annie L., Fort Valley State College
LeFlore, William B., Spelman College
Race, James, Jr., Texas Southern University
Tounsel, James G., Virginia State College
Walker, Charles A., Tuskegee Institute

4. Fellowships and Scholarships, 1971

The Crocker Scholarship:

Elie Mousalli, Ecology Course
Verena Gauss, Botany Course

The Jacobs Scholarship:

Jean-Luc Popot, Physiology Course
Eugene G. Sacevich, Physiology Course

The Memhard Scholarship:

Donald A. Colbert, Embryology Course

5. Training Programs

Fertilization and Gamete Physiology Research Training Program

I. Instructors

Charles B. Metz, University of Miami, Program Chairman
Marco Crippa, Laboratorio di Embriologia Molecolare, Naples, Italy
Gertrude W. Hinsch, University of Miami
Lajos Piko, Veterans Administration Hospital, Sepulveda, California
Allen W. Schuetz, Johns Hopkins University
William H. Telfer, University of Pennsylvania

II. Laboratory Assistants

Mrs. Carolyn Conway, Electron Microscope Assistant
Mr. Steve Senft, Photographic Assistant
Mrs. Angela O'Rand, Program Secretary

III. Trainees

Arakelian, Helen, Michigan State University
Bantle, John A., Ohio State University
Bergstrom, Beverly H., University of North Carolina
Bischoff, William L., University of North Carolina
Cayer, Marilyn L., University of Miami
Clegg, Kerry B., University of California, Los Angeles
Conway, Arthur F., University of Miami
Cooper, Alan Douglas, Worcester State College
Ecklund, Peter S., Wayne State University
Gould, Stanley F., Wayne State University
Howe, Craig, King's College, University of Cambridge, England

Kahn, James L., University of Toledo
LaMarca, Michael J., Lawrence University
Mann, William J., Jr., Pennsylvania State College of Medicine
Matsumoto, Lloyd H., St. Louis University
Shippee, Elizabeth, Cornell University
Wassarman, Paul M., Purdue University

IV. Lectures

W. H. Telfer	Function of nurse cells and follicular epithelium in insect ovaries
G. W. Duncan	The effects of prostaglandin on reproductive functions
M. Crippa	Mechanism for ribosomal gene amplification
F. H. Bronson	Pheromones and mammalian reproduction
B. G. Brackett	*In vitro* fertilization of mamalian ova
K. A. Lawrence	Antibodies to gonadotropins and their effect on gonadotropin action and fertility
D. W. Fawcett	The organization of the mammalian seminiferous epithelium
I. Dawid	The function of mitochondrial DNA in frog eggs and embryos
D. N. Ward	The amino acid sequence of ovine and bovine luteinizing hormone

V. Special Lectures

Muriel H. Walker	The arrangement of nucleoprotein in elongate sperm heads
John Biggers	Metabolic changes in early mammalian development
Yosef Aloni	Transcription of mitochondrial DNA in HeLa cells

Excitable Membrane Physiology and Biophysics Training Program

I. Instructors

W. J. Adelman, Jr., Director, Professor of Physiology, University of Maryland School of Medicine
C. Armstrong, Professor, University of Rochester School of Medicine
J. W. Moore, Professor of Physiology, Duke University School of Medicine
T. Narahashi, Professor of Physiology, Duke University School of Medicine
Y. Palti, Associate Professor of Physiology, The Technion Medical School, Haifa, Israel

II. Consultants

K. S. Cole, Senior Research Biophysicist, NINDS, National Institutes of Health
L. J. Mullins, Professor of Biophysics, University of Maryland School of Medicine

III. Trainees

Borg, Dr. Sidney F., Stevens Institute of Technology
Brutsaert, Dr. Dirk L., University of Antwerp
Fan, Dr. Chungpeng, Rutgers University
Froelich, Mr. Otto, University of Toledo
Gardner, Dr. Colin R., Harvard Medical School
Goldfinger, Mr. Melvin D., University of Maryland Medical School
Griffin, Patricia M., University of Pennsylvania
Harbus, Mr. Frederick I., Massachusetts Institute of Technology
Michaels, Mr. David W., University of Delaware
Rose, Dr. Mary C., Duke University
Sidie, Dr. James M., Jr., Indiana University
Starzak, Dr. Michael E., State University of New York at Binghamton

IV. LECTURES

T. SCHWARTZ	The flux equation—its rationale and relation to diffusion regimes
	The Ussing-Teorell "Unidirectional Flux Equation"
	The Gibbs-Donnan equilibrium
	The Goldman equation—with and without active transport and/or constant field conditions
	Simple diffusion regimes—relation to electric equivalent circuits
N. C. HEBERT	Properties of ion sensing glass micro-electrodes
S. WATSON	Evolution of structure in membranes of nitrifying bacteria
K. S. COLE	Biophysics and a nerve impulse
	Voltage clamp strategy
J. W. MOORE	Voltage clamp tactics
	Voltage clamp arrangements
	Ionic current kinetics
Y. PALTI	The Hodgkin-Huxley axon
	Action potential reconstruction and propagation
	Varying potential voltage clamps
W. K. CHANDLER	General properties of internally perfused atoms
	Perfusion of axons with solutions of low ionic strength
	Ionic selectivity of the axonal membrane
	Properties of the delayed rectifier
	Sodium conductance with K-free fluoride solutions inside the axon
L. COHEN	Birefringence and fluorescence changes during axon activity
	Light scattering changes in axons
C. ARMSTRONG	The K channels of nerve and other excitable tissues
	Interactions of TEA and TEA derivatives with the K channels, and what this tells about the nature of the channels I.
	Interactions of TEA and TEA derivatives with the K channels, and what this tells about the nature of the channels II.
G. EHRENSTEIN	Artificial membranes. I. Structure
	Artificial membranes. II. Specificity
	Artificial membranes. III. Excitability
I. TASAKI	Macromolecular approach to nerve excitation
	Fluorescence studies of nerve excitation
W. STOECKENIUS	Membrane structure I.
	Membrane structure II.
	Membrane structure discussion
H. LECAR	Noise and fluctuations in nerve membranes
L. MULLINS	Recovery processes in axons-active ion transport
	The interaction between diffusion and chemical forces in determining the resting membrane potential
	Inhibitors of Na^+ and K^+ currents-inferences regarding mechanisms
	Models for nerve excitation
D. GILBERT	Membrane surface charges I.
	Membrane surface charges II.
W. ADELMAN	Properties of the periaxonal space in modifying membrane behavior
	Properties of the perineuronal space in modifying neuronal behavior
F. A. DODGE	Receptor potentials
T. NARAHASHI	Mode of action of drugs on excitable membranes. I. General consideration and tetrodotoxin
	Mode of action of drugs on excitable membranes. II. Toxins, anesthetics, insecticides and enzymes
	Characteristics of end-plate membrane conductances
H. GRUNDFEST	Electrically excitable membranes
	Electrically inexcitable membranes
A. B. STEINBACH	Neuromuscular transmission. I. Presynaptic transmitter release
	Neuromuscular transmission. II. Postsynaptic response

6. Tabular View of Attendance, 1967–1971

	1967	*1968*	*1969*	*1970*	*1971*
INVESTIGATORS—TOTAL	590	528	566	532	554
Independent	313	281	310	324	322
Library Reader	78	76	68	73	76
Research Assistants	199	171	188	135	156
STUDENTS—TOTAL	132	122	118	142	130
Invertebrate Zoology	41	39	35	41	29
Embryology	20	20	20	28	28
Physiology	31	30	30	31	33
Experimental Botany	20	15	16	19	22
Ecology	20	18	17	23	18
TRAINEES—TOTAL	16	17	29	33	44
TOTAL ATTENDANCE	738	667	713	707	728
Less Persons represented in two categories	4	7	5	0	0
	734	660	708	707	728
INSTITUTIONS REPRESENTED—TOTAL	177	169	187	191	219
FOREIGN INSTITUTIONS REPRESENTED	29	23	24	21	27

7. Institutions Represented, 1971

Agnes Scott College
Albert Einstein College of Medicine
American Museum of Natural History
Amherst College
Barnard College
Bennington College
Boston City Hospital
Boston College
Boston University
Boston University School of Medicine
Brandeis University
Brooke Army Medical Center
Brooklyn College, The City University of New York
Brown University
Bucknell University
California, University of, Berkeley
California, University of, Davis
California, University of, Irvine
California, University of, Los Angeles
California, University of, Riverside
California, University of, San Diego
California, University of, Santa Barbara
California, University of, Santa Cruz
California Institute of Technology
Cambridge, University of
Carnegie Institution of Washington
Carnegie-Mellon University
Case Western Reserve University
Chicago, University of
Cincinnati, University of
City College, The City University of New York
Clark University
Colby College
College of William and Mary
Colorado, University of
Colorado, University of, Medical Center
Columbia University
Columbia University, College of Physicians and Surgeons
Connecticut, University of
Connecticut, University of, Health Center
Connecticut, University of, Medical School
Cornell University
Cornell University Medical College
Dartmouth College
Dartmouth Medical School
Davidson College
Delaware, University of
Douglass College
Drew University
Drexel University
Duke University
Duke University Medical Center
East Stroudsburg State College
Emory University
Florida, University of
Florida Atlantic University
Florida State University
Goucher College
Georgetown University

Hamilton College
Harvard Medical School
Harvard University
Hawaii, University of
Hunter College, The City University of New York
Idaho, University of
Illinois, University of
Illinois Institute of Technology
Immaculata College
Indiana University
Institute for Basic Research in Mental Retardation
Institute for Cancer Research, The
Institute for Muscle Research, Inc.
Iowa, University of
Iowa State University
Johns Hopkins University, The
Johns Hopkins University, The, School of Medicine
Juniata College
Kansas, University of
Kansas State University
Kent State University
Kentucky, University of
Keuka College
Lawrence University
Lehman College, The City University of New York
Long Island University
Louisiana State University
Maine, University of
Marquette University
Marine Research Foundation, Inc.
Maryland, University of
Maryland, University of, School of Medicine
Massachusetts, University of
Massachusetts General Hospital
Massachusetts Institute of Technology
Medical College of Ohio at Toledo
Medical College of Pennsylvania
Mellon Institute of the Carnegie-Mellon University
Miami, University of
Miami University
Michigan, University of
Michigan State University
Millersville State College
Milton S. Hershey Medical Center
Minnesota, University of
Mississippi State College for Women
Mount Holyoke College
Mount Sinai School of Medicine, The City University of New York
National Institute of Mental Health
National Institutes of Health
National Marine Fisheries Service
New Hampshire, University of
New Mexico, University of
New Mexico, University of, School of Medicine
New York Blood Center, The
New York Medical College
New York University College of Dentistry
New York University Medical College
North Carolina, University of
North Carolina State University of Raleigh
Northwestern University
Notre Dame, University of
Oak Ridge National Laboratory
Oakland University
Oberlin College
Ohio State University
Ohio University
Oregon, University of
Pennsylvania, University of
Pennsylvania, University of, School of Medicine
Pennsylvania Hospital
Pennsylvania State University
Pittsburgh, University of
Pomona College
Princeton University
Puget Sound, University of
Purdue University
Queens College, The City University of New York
Reed College
Rhode Island, University of
Rice University
Rochester, University of
Rochester, University of, Medical School
Rockefeller University, The
Rutgers University
Rutgers University Medical School
St. Elizabeth Hospital
St. Louis University
St. Vincent College
St. Vincent's Hospital and Medical Center of New York
San Fernando Valley State College
Scripps Institution of Oceanography
Smith College
South Florida, University of
Southern California, University of, Medical School
Stanford University
State University of New York, Downstate Medical Center
State University of New York, Upstate Medical Center
State University of New York at Albany
State University of New York at Buffalo
State University of New York at Stony Brook
Syracuse University
Temple University

Tennessee, University of
Texas, University of
Texas, University of, at Austin
Texas Christian University
Toledo, University of
Trinity College
Tufts University
Tulane University
Union College
Upsala College
Utah, University of
Vassar College
Vermont, University of
Veterans Administration Hospital, Brooklyn
Veterans Administration Hospital, California
Virginia, University of
Virginia, University of, School of Medicine
Wake Forest University
Washington, University of
Washington University
Washington University School of Medicine
Wayne State University
Wesleyan University
Williams College
Wisconsin, University of
Wistar Institute
Woods Hole Oceanographic Institution
Worcester State College
Yale University
Yale University School of Medicine

FOREIGN INSTITUTIONS REPRESENTED, 1971

American University of Beirut, Lebanon
Antwerp, University of, Belgium
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Toronto, University of Canada
Trondheim, University of, Norway
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8. FRIDAY EVENING LECTURES, 1971

July 2	
PRESTON CLOUD University of California Santa Barbara	Biospheric, atmospheric and crustal evolution of the primitive earth
July 8	
AHARON KATCHALSKY Weizmann Institute of Science Alexander Forbes Lecturer at MBL	Biothermodynamics and network analysis part I
July 9	
AHARON KATCHALSKY	Biothermodynamics and network analysis part II
July 16	
R. S. WOLFE University of Illinois	Methyl transfer reactions in methane bacteria and their ecological significance

July 23

Harry Eagle Albert Einstein College of Medicine W. J. V. Osterhout Memorial Lecture	pH, contact inhibition and cell metabolism

July 30

Melvin J. Cohen Yale University	Some functional implications of neuronal geometry

August 6

O. L. Miller, Jr. Oak Ridge National Laboratory	Visualization of genes in action

August 13

Seymour Benzer California Institute of Technology	Genes and the nervous system of *Drosophila*

August 20

R. K. Selander University of Texas Rand Fellow at the MBL	Biochemical polymorphism and systematics

August 27

Martin Raff National Institute for Medical Research, London Lillie Fellow at the MBL	An immunological approach to lymphocytes and the cell surface

9. Tuesday Evening Seminars, 1971

August 10

Charles B. Metz Alberto Seiguer Amalia Castro	Mammalian sperm hyaluronidase, an isoantigen of possible interest for fertility control
Gertrude W. Hinsch Muriel H. Walker	Spermatocyte formation in the vas deferens of spider crabs
Muriel H. Walker Gertrude W. Hinsch	Microsporidia in the reproductive tract of *Libinia dubia*
Leonard Nelson	Motility control mechanisms in *Arbacia* sperm

August 17

Richard L. Miller	*Trichoplax adhaerens* Schulze, 1883: return of an enigma
Seymour Zigman	Effects of near UV tryptophan photoproducts on proteins
T. A. Borgese Richard Egnor	Iso-electric gel focusing of duck hemoglobins
Laslo Z. Bito David Turansky Alice Van Voris	Concentrative accumulation of prostaglandins by some tissues of marine invertebrates and vertebrates

10. Members of the Corporation, 1971

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V. REPORT OF THE LIBRARIAN

The MBL Associates' gift for 1971 was $10,507 and the Library had the good fortune to be the sole recipient of their gift. This will make a tremendous difference in our "book" section, as we have been unable to add to this area due to cutbacks in the budget. A number of committees were formed in 1970 for the purpose of recommending books that were essential to the MBL and many lists were prepared by the scientists. The Associates' gift will cover the cost of all the recommended books.

In December we moved the reprints down to the basement stack where there is no room for expansion but the collection remains intact. What was the reprint floor now houses all journal titles from *Proceedings of the National Academy of Sciences; India* through to the end of the alphabet. The rest of the journal collection covers two and a half floors and there is now room for expansion for the next seven years. It took six of us (four from the Library and two from Buildings and Grounds) five weeks to move approximately 140,000 volumes. While we were making the major move we changed the arrangement of the titles so that the articles are no longer used in the title when arranged alphabetically. Journals are now easier to find, one does not have to remember if the title has the words "the," "de la," *etc.*

The G. K. Hall Company published our catalog in 12 volumes and to date over 70 sets have been sold. The MBL received a 10 per cent royalty fee after the first 35 sets have been sold.

Xerox continues to be one of our major projects. We received over 4,400 requests from other libraries during the year. There are now two xerox machines in the library throughout the year and during the summer months they run constantly.

In 1971 we had 4,146 serial titles, 2,476 received currently. Our holdings now total 146,158 volumes.

VI. REPORT OF THE TREASURER

The market value of the General Endowment Fund and the Library Fund at December 31, 1971, amounted to $2,294,949 and the corresponding securities are entered in the books at a value of $1,555,338. This compares with values of $2,197,603 and $1,574,735, respectively, at the end of the preceding year. The average yield on the securities was 4.15% of the market value and 6.13% of the book value. Uninvested principal cash was $7,757. Classification of the securities held in the Endowment Fund appears in the Auditor's Summary of Investments.

The market value of the Pooled Securities at December 31, 1971, amounted to $870,538 as compared to book values of $667,280. These figures compare with values of $770,487 and $662,428, respectively, at the close of the preceding year. The average yield on the securities was 3.53% of the market value and 4.61% of the book value. Uninvested principal cash was in the amount of $2,295.

The proportionate interest in the Pool Fund Account of the various funds, as of December 31, 1971, is as follows:

Pension Funds	26.12%
General Laboratory Investment	19.83%
F. R. Lillie Memorial Fund	2.16%
Anonymous Gift	.74%

Other:

Bio Club Scholarship Fund	.56%
Rev. Arsenius Boyer Scholarship Fund	.68%
Gary N. Calkins Fund	.65%
Allen R. Memhard Fund	.12%
Lucretia Crocker Fund	2.35%
E. G. Conklin Fund	.40%
Jewett Memorial Fund	.20%
M. H. Jacobs Scholarship Fund	.28%
Herbert W. Rand Fellowship	20.00%
Mellon Foundation	9.44%
Mary Rogick Fund	2.07%
Swope Foundation	5.20%
Clowes Fund	9.20%

Donations from MBL Associates for 1971 amounted to $10,507 as compared with $9,724 for 1970. Unrestricted gifts from foundations, societies and companies amounted to $32,947.

During the year we administered the following grants and contracts:

Investigators	*Training*	*MBL Institutional*
5 NIH	4 NIH	1 NIH
4 NSF	2 NSF	1 NSF
1 ONR	1 Sloan	1 AEC
1 WHIH		
1 EPA		
1 MWPC		
13	7	3

The majority of federally funded grants and contracts provided for reimbursement of indirect costs on a cost per square foot basis, for the laboratory space assigned to a particular research project. A provisional rate of $10.00 per square foot is still in effect. The space cost basis is also applicable to NSF training grants, but actual funding was at a considerably lower level due to limited granting agency funds. Indirect costs for NIH training grants are computed at a rate of 8% of allowable direct costs.

The following is a statement by the Auditors:

To the Trustees of Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheet of Marine Biological Laboratory as of December 31, 1971, the related statements of operating expenditures and income and funds for the year then ended. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances. We previously examined and have reported on 1970 financial statements.

In our opinion, the aforementioned financial statements (pages 87 to 91) present fairly the financial position of Marine Biological Laboratory at December 31, 1971 and 1970 and the results of its operations for the years then ended and the changes in funds for the year ended December 31, 1971 in conformity with the accounting principles referred to in Note A to the financial statements applied on a consistent basis.

The supplementary schedules (pages 92 to 93) included in this report were obtained from the Laboratory's records in the course of our examination and, in our opinion, are fairly stated in all material respects in relation to the financial statements, taken as a whole.

Boston, Massachusetts
March 30, 1972 LYBRAND, ROSS BROS. AND MONTGOMERY

MARINE BIOLOGICAL LABORATORY

BALANCE SHEET

December 31, 1971 and 1970

Investments

	1971	*1970*
Investments held by Trustee:		
Securities, at cost (approximate market quotation, 1971—$2,294,949; 1970—$2,173,414)	$ 1,555,338	$ 1,573,362
Cash	7,757	2,641
	1,563,095	1,576,003
Investments of other endowment and unrestricted funds:		
Pooled investments, at cost (approximate market quotation, 1971—$870,538; 1970—$770,487) less $5,728 temporary investment of current fund cash	661,552	656,700
Other investments	725,151	1,125,150
Cash	2,295	485
Due from current fund	94,412	65,176
	$ 3,046,505	$ 3,423,514

Plant Assets

Land, buildings, library and equipment	12,443,510	9,662,611
Less allowance for depreciation (Note A)	2,161,247	1,899,406
	10,282,263	7,763,205
Construction in progress	—	2,476,261
Investments at cost (approximate market quotation, 1971—$537,115; 1970—$536,875)	737,881	712,745
	$11,020,144	$10,952,211

Current Assets

Cash	199,877	276,166
Temporary investment in pooled securities	5,728	5,728
Accounts receivable (U. S. Government, 1971—$69,395; 1970—$52,621)	248,396	147,881
Inventories of supplies and bulletins	44,999	41,749
Other assets	7,798	10,310
Due to endowment funds	(94,412)	(65,176)
	$ 412,386	$ 416,658

MARINE BIOLOGICAL LABORATORY

BALANCE SHEET

December 31, 1971 and 1970

Invested Funds

	1971	*1970*
Endowment funds given in trust for benefit of the Marine Biological Laboratory	$ 1,563,095	$ 1,576,003
Endowment funds for awards and scholarships:		
Principal	427,702	427,702
Unexpended income	55,591	51,458
	483,293	479,160
Unrestricted funds functioning as endowment	779,190	1,179,190
Retirement fund	279,407	246,833
Pooled investments—accumulated loss	(58,480)	(57,672)
	$ 3,046,505	$ 3,423,514

Plant Funds

Funds expended for plant, less retirements	12,443,510	11,797,632
Less allowance for depreciation charged thereto	2,161,247	1,899,406
	10,282,263	9,898,226
Accounts payable	—	341,240
Unexpended plant funds	737,881	712,745
	$11,020,144	$10,952,211

Current Liabilities and Funds

Accounts payable and accrued expenses	31,485	10,309
Advance subscriptions	34,338	34,275
Unexpended grants—research	47,837	66,324
Unexpended balances of gifts for designated purposes	39,725	25,020
Current fund	259,001	280,730
	$ 412,386	$ 416,658

The accompanying note is an integral part of the financial statements.

Note A.—*Accounting Principles:* The following accounting principles have been reflected in the accompanying financial statements:

1. Investments are stated at cost.
2. Investment income is recorded on a cash basis.
3. Operating income is recorded when earned.
4. Expenses are recorded on an accrual basis.
5. Depreciation has been provided for plant assets at annual rates ranging from 1% to 5% of the original cost of the assets.

MARINE BIOLOGICAL LABORATORY

STATEMENT OF OPERATING EXPENDITURES AND INCOME

Years Ended December 31, 1971 and 1970

	1971						
	Salaries and Wages	*Other Costs and Expenses*	*Depreciation (Note A)*	*Total*	*Charged to Grants*	*1971 Total*	*1970*
Operating Expenditures:							
Instruction		$ 12,116	$ 74,803	$ 86,919	$331,396	$ 418,315	$ 272,413
Research		40,387	83,788	124,175	335,462	459,637	479,970
Dormitories	$ 16,913	48,074	80,441	145,428		145,428	93,519
Dining		89,637		89,637		89,637	64,978
Library	34,811	15,453	18,016	68,280		68,280	64,934
Back sets, serials and binding		49,707		49,707		49,707	43,882
Biological Bulletin	5,414	43,928		49,342		49,342	41,124
Support services:							
Apparatus	53,771	60,320		114,091		114,091	80,903
Supply	80,831	58,945	5,303	145,079		145,079	148,969
Administration	97,853	85,155		183,008		183,008	165,159
Plant operation	154,077	121,351	2,182	277,610		277,610	270,799
Grant expenditures for support services					8,130	8,130	47,483
Other		34,800		34,800		34,800	33,357
	$443,670	$659,873	$264,533	1,368,076	$674,988	2,043,064	1,807,490

MARINE BIOLOGICAL LABORATORY

STATEMENT OF OPERATING EXPENDITURES AND INCOME CONTINUED

Years Ended December 31, 1971 and 1970

	Fees	*Other*	*Total*	*Charged to Grants*	*1971 Total*	*1970*
Income:						
Instruction	$ 69,600		69,600	$331,396	400,996	251,120
Research	214,231		214,231	335,462	549,693	532,346
Dormitories		$146,559	146,559		146,559	99,633
Dining		101,419	101,419		101,419	78,828
Library	24,280	34,359	58,639		58,639	50,203
Biological Bulletin		56,852	56,852		56,852	54,715
Support services:						
Apparatus		45,847	45,847		45,847	36,592
Supply		68,058	68,058		68,058	62,379
Administration		19,103	19,103		19,103	16,101
Investments income		131,615	131,615		131,615	183,787
Gifts used for current expense		52,532	52,532		52,532	38,690
Allowance for indirect costs		63,362	63,362		63,362	62,666
Grants for general support					—	48,103
Grants for support services				8,130	8,130	47,483
Other		1,670	1,670		1,670	1,925
	$308,111	$721,376	1,029,487	$674,988	1,704,475	1,564,571
Excess of current expenditures and depreciation over current income			338,589		338,589	242,919
Reduction in plant funds for depreciation			264,533		264,533	167,423
Excess current expenditures			$ 74,056		$ 74,056	$ 75,496

The accompanying note is an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

Statement of Funds

Year Ended December 31, 1971

	Balance December 31, 1970	*Gifts and Other Receipts*	*Investment Income*	*Used for Current Expenses*	*Other Expenditures*	*Balance December 31, 1971*
Invested funds	$3,423,514	$ 37,054 (400,000) (2)	$155,420	$131,366	$ 38,117	$3,046,505
Unexpended plant funds	$ 712,745	309,911	24,792		309,567	$ 737,881
Unexpended research grants	$ 66,324	722,145		740,632		$ 47,837
Unexpended gifts for designated purposes	$ 25,020	25,242		10,507	30	$ 39,725
Current fund	$ 280,730	(74,056) (1) 400,000 (2)			347,673	$ 259,001
		$1,020,296	$180,212	$882,505	$695,387	
Gifts and grants for facilities construction		309,911				
Other gifts and receipts		25,242				
Grants for research, training and support		722,145				
Appropriated from current income and other		37,054				
(1) Excess of current expenditures over income		(74,056)				
(2) Transfer from invested funds		—				
		$1,020,296				
Expended for new laboratory and dormitory—dining hall					657,240	
Scholarship awards					11,115	
Payments to pensioners					10,672	
Loss on sale of securities					16,330	
Other					30	
					$695,387	

The accompanying note A is an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1971

	Cost	*Per-cent of Total*	*Market Quotations*	*Per-cent of Total*	*Investment Income 1971*
Securities held by Trustee:					
General endowment fund:					
U. S. Government securities	$ 25,065	2.0	$ 26,070	1.4	$ 1,803
Corporate bonds	640,163	51.4	565,180	30.0	36,203
Preferred stocks	84,770	6.8	61,318	3.3	3,385
Common stocks	495,848	39.8	1,231,644	65.3	35,313
	1,245,846	100.0	1,884,212	100.0	76,704
General educational board endowment fund:					
U. S. Government securities	51,112	16.5	53,183	12.9	3,698
Other bonds	146,965	47.5	118,592	28.9	9,590
Preferred stocks	15,476	5.0	7,024	1.7	579
Common stocks	95,939	31.0	231,938	56.5	4,763
	309,492	100.0	410,737	100.0	18,630
Total securities held by Trustee	$1,555,338		$2,294,949		95,334
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government securities	96,397	14.4	98,167	11.2	7,194
Corporate bonds	89,200	13.4	73,686	8.5	5,299
Preferred stocks	60,158	9.0	56,550	6.5	3,145
Common stocks	421,525	63.2	642,135	73.8	15,095
	667,280	100.0	$ 870,538	100.0	30,733
Less temporary investment of current fund cash	5,728				249
	661,552				30,484
Other investments:					
U. S. Government securities	27,938				1,133
Other bonds	15,029				750
Common stocks	49,635				2,823
Real estate	17,549				
Short-term commercial notes	615,000				35,485
	725,151				40,191

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS CONTINUED

December 31, 1971

	Cost	*Investment Income 1971*
Total investments of other endowment and unrestricted funds	$1,386,703	70,675
Total		166,009
Custodian's fees charged thereto		10,589
Investment income distributed to invested funds		155,420
Plant investments:		
Federal agency and corporate bonds	140,000	6,252
Common stock	595,010	18,436
Preferred stock	2,871	104
	$ 737,881	24,792
Current investments:		
Temporary investment in pooled securities	$ 5,728	249
Total investment income		$180,461

Reference: *Biol. Bull.*, **143**: 94-111. (August, 1972)

THE SENSORY BASIS OF HOST RECOGNITION BY SYMBIOTIC SHRIMPS, GENUS *BETAEUS*[1]

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Animals living in symbioses serve as excellent material for the analysis of external stimuli controlling adaptive behavior. To a mobile partner, the host organism represents a principal source of environmental stimuli, a source easily manipulated by the investigator. Experimental analyses of symbiotic relationships have demonstrated that chemical substances of host origin elicit host-oriented behavior in crustacean species associated with pelecypods (Sastry and Menzel, 1962), polychaetes (Carton, 1968; Davenport, Camougis and Hickok, 1960), echinoids (Gray, McClosky and Wiehe, 1968), and amphinurans (Webster, 1968). These studies focused primarily on the role of chemical stimuli in effecting the respective symbiotic relationships. In surveying earlier work on crustacean orientation, however, Pardi and Papi (1961) note that even such relatively simple behavioral responses as kinetic and tactic orientation appear to be governed by higher neural centers, sometimes utilizing information from multiple sensory modalities. More recently, the interaction of multi-modal stimuli has been demonstrated to elicit and direct feeding behavior in several species of decapod crustaceans (Hiatt, 1948; Symons, 1964; Hazlett, 1968).

The carideans *Betaeus harfordi* (Kingsley) and *Betaeus macginitieae* Hart are two of five species of betaeid shrimps adapted to a symbiotic existence (Hart, 1964). *Betaeus harfordi* occurs in the mantle cavity of all eight species of California abalone, *Haliotis* spp. (Cox, 1962; Hart, 1964). *B. macginitieae* associates predominantly with the homochromous giant red sea urchin, *Strongylocentrotus franciscanus* (Agassiz) and occasionally with the purple urchin, *S. purpuratus* (Simpson) (Ache, 1970; Hart, 1964). Laboratory observations indicate that adult shrimps of both species leave their hosts during dark periods and return directly from distances up to 1 m away within a few minutes of the onset of light. (Ache, 1970). In doing so, they provide a behavioral response, *i.e.,* tactic locomotion toward the host, suitable for analysis of the stimuli mediating distant host recognition.

The present investigation attempts to elucidate and compare the sensory bases of the host location behavior of *B. harfordi* and *B. macginitieae* and to explain the apparent specificity of their respective relationships in terms of the sensory competence of the shrimps.

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[1] Conducted under Contract NONR 4222(03) with the Office of Naval Research and Grant No. NB04372 from the United States Public Health Service.

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MATERIAL AND METHODS

Organisms

Specimens of *Betaeus harfordi* and *B. macginitieae,* 1.0–3.5 cm total length, were collected along with their respective hosts, the abalones, *Haliotis corrugata* Gray, *H. rufescens* Swainson, and *H. cracherodii* Leach and the urchin, *Strongylocentrotus franciscanus,* from subtidal populations in the Santa Barbara area. Shrimps were maintained in the laboratory isolated from their hosts on a diet of frozen *Artemia.* Shrimps were utilized for experimentation between the 2nd and 10th days of holding. Hosts or other organisms to be tested as potential sources of stimuli (test organisms) were held without feeding and utilized within three days of laboratory confinement.

Apparatus

Two types of choice apparatus were utilized to quantify the host-oriented behavior of *Betaeus.* One apparatus was simply a large (1.0 × 1.3 × 0.2 m) seawater-filled rectangular tank or arena. Seawater was continuously introduced via four inlet tubes, one located in each corner of the tank, and maintained at a

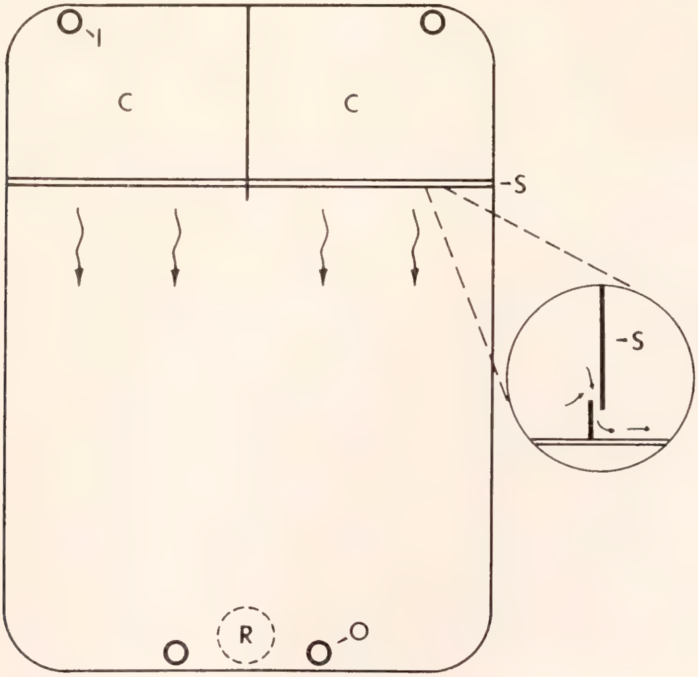

FIGURE 1. Diagram (top view) of two-celled choice apparatus: C—test or control cells; I—seawater inlets; O—seawater outlets; S—transparent plexiglass screen; R—removable release cylinder. Arrows indicate direction of flow into choice area. Inset is transverse view of continuous opening along bottom of plexiglass screen. Arrows indicate flow through this opening.

depth of 15 cm by two clear plastic standpipe drains centered on the longer axis of the tank. Plastic-coated screens fitted into each of the corners served to confine test organisms within approximately 15 cm of the seawater inlets, yet did not retard the movement of shrimps into or out of the corner compartments. A removable length of 14 cm diameter clear plastic tubing centered in the tank served as a release point for shrimps. The overhead fluorescent lamps of the room supplied relatively uniform illumination to the apparatus.

Shrimp behavior was also quantified in a 2-celled choice apparatus designed to be compatible with the fast-moving *Betaeus* yet retain the binomial simplicity of a conventional Y maze. The apparatus (Fig. 1) incorporated a 33 × 46 × 12 cm opaque white polyethylene pan fitted with a T-shaped transparent plastic divider to form two small compartments (herein referred to as test and control cells) and a larger compartment (herein referred to as the choice area). A baffled opening in the transverse partition (inset, Fig. 1) allowed seawater introduced into the test and control cells to flow into the choice area where it was removed by two constant-level siphons. An input of 7.0 ml/sec of new seawater to each cell produced an even, laminar flow of approximately 5 mm/sec along the bottom of the choice area (arrows, Fig. 1). A removable opaque cylinder allowed introduction of single shrimp into the choice area with minimal directional bias. A 7.5w frosted incandescent lamp centered over the apparatus 50 cm from the water's surface supplied even, low-intensity illumination to the choice area. The 2-celled choice apparatus was adapted for detailed analysis of visual stimuli by replacing the open-bottomed transparent partition with a watertight transparent partition to insure chemical isolation of all three compartments and by removing the seawater inlet and outlet tubes to create a static system. A 4 cm strip of opaque white plastic attached to the transparent panel to increase the separation between the two cells enhanced the resolution of right and left choices.

Procedure and data analysis

Shrimps were isolated from their hosts up to 10 days prior to testing. Symons (1964) has noted that the ability of either chemical or tactile stimuli to evoke feeding in the crab *Hemigrapsus oregonensis* increases over a 10 day period of starvation, approaching the ability of combined tactile-chemical stimuli to elicit the same response by day 10. To minimize any bias due to threshold change, all our experiments within a series were performed in as short a time as possible.

Arena apparatus—Ten minutes prior to introduction of the shrimps two to four host organisms were placed in each of two diagonally opposite corner compartments. As controls, three non-host organisms, the seastar *Dermasterias imbricata*, were placed in each of the two remaining corner compartments. The presence of other live organisms in the control compartments minimized the possibility of the data reflecting a generalized response to any animate object. The animal complement of each compartment was equated by weight (± 50 g) to the mean weight of three seastars, 320 g. Twelve or 15 shrimp selected from a group of 120–125 individuals were dip-netted into the central release cylinder, held for 5 minutes, and then released by removing the cylinder. The location of each individual was recorded 30 minutes following release. This sequence was repeated eight times for any one set of test conditions, with the contents of the corner compartments moved clockwise one compartment with each repetition. Mucus and debris were wiped from each compartment with each rotation.

The hypothesis that the terminal distribution of shrimp was randomly divided between the two pair of corner compartments was tested by comparing the total number of shrimps found within the two host-containing compartments with the number found within the two control compartments. In each experiment, the probability of obtaining the observed distribution was tested for its association with a theoretical distribution of 0.50–0.50, utilizing chi-square.

Two-celled choice apparatus.—Experimental protocol with this apparatus consisted of introducing potential sources of stimuli to one or both of the cells, allowing 10 minutes for equilibration, and monitoring the time ($t \leqq 6$ min) required for each of 30 shrimps, individually and sequentially introduced, to leave the release point, traverse the length of the choice area, and contact the transparent partition delimiting one of the two cells. Thirty shrimps, selected at random from groups of 70–80 individuals, were utilized in each experiment. Experiments comprising an experimental series utilized the same group of 70–80 shrimps. A new group of 70–80 shrimps was obtained for each experimental series. The contents of the two cells were exchanged in each experiment after testing one-half of the 30 shrimps, the exchange being accompanied by washing and refilling of the apparatus with fresh seawater. Data displaying a significant non-experimental bias ($P < 0.05$) to either cell were voided and the experiment repeated. This practice, which required repetition of approximately 8% of the experiments, effectively controlled for transient bias (*e.g.*, obstruction of an inlet tube by particulate material) that may have occurred during the course of an experiment.

The number of shrimps reaching criterion, *i.e.*, those contacting the transparent partition delimiting the host-containing cell, was compared by chi-square analysis to an expected distribution in which 50% of the total number of individuals making a choice go to each cell. This value is subsequently referred to as "χ^2 choice." In many experiments, a number of individuals failed to move, or move but failed to reach criterion within the 6 minute experimental period. For an experiment questioning the relative attractiveness of a stimulus situation, the number of organisms not reaching criterion represents significant information. Formulation of this category involves combining the number of individuals not moving, those moving but not reaching criterion, and those choosing the control cell. Although detailed *a priori* knowledge of how shrimps making a choice relate to those not stimulated to move remains unknown, individuals of all three combined groups can be considered as not displaying a positive response to the stimulus source of a particular experiment. This rationale is not without precedent (see Davenport, 1950). Differences in the numbers of shrimps failing to reach criterion for any two experiments of a series were tested for significance with a two-way contingency analysis adjusted for continuity. (Simpson, Roe, and Lewontin, 1960, page 190).

RESULTS

The Betaeus-Haliotis *association*

Experiments utilizing arena apparatus. These experiments attend to the question: Do shrimps collected from the host, *H. rufescens*, require both chemical and

TABLE I

Arena experiments: B. harfordi-H. rufescens vs. D. imbricata

Experiment	Stimulus modality removed	Shrimps tested	Shrimps choosing		χ^2 choice	*P*
			Host	Control		
1	None	96	82	8	72.3	<0.005
2	Chemical	111	29	38	0.12	0.50–0.75
3	Visual	111	78	25	27.2	<0.005
4	None, hosts in all corners	96	34	48	2.38	0.10–0.25

visual stimuli of host origin to effect host location? Shrimps were permitted to choose between two corner compartments containing host abalone and two containing the control organism while visual or chemical stimuli were selectively removed from the choice situation. Table I summarizes the results of these experiments which extended over an eight day period. With both visual and chemical stimuli present, shrimps preferentially selected those compartments containing abalone, *H. rufescens*, over those containing the seastars (Experiment 1). With all organisms contained in clear glass 4 liter beakers to eliminate chemical stimuli from the choice situation, but otherwise identical protocol maintained, the differential response elicited in Experiment 1 was abolished (Experiment 2). However, with the corner compartments covered with eight layers of white cheesecloth so as to exclude visual stimuli from the choice situation yet not alter the flow characteristics of the system, shrimps exhibited preferential selection of the abalone-containing compartments (Experiment 3). To further eliminate the possibility that bias existed in the experimental procedure itself, host abalones were substituted for seastars in the control compartments, *i.e.*, all compartments contained host abalone, and Experiment 1 was repeated (Experiment 4). No significant difference occurred in the number of shrimps selecting either of the two pair of host-containing compartments.

Testing shrimps in groups introduced the possibility of bias due to shrimp-shrimp interactions. The 30 minute experimental period allowed multiple responses by any one shrimp, which were observed to occur in a small percentage of the trials. For these reasons, and since more than 50% of the shrimps entered a corner compartment within the first 3–4 minutes of the 30 minute experimental period, further analysis utilized the 2-celled choice appartus, designed for short term observation of individual organisms.

Experiments utilizing 2-celled choice apparatus. The question considered in the preceding group of experiments was again tested as a basis for comparison of the two techniques. In these experiments, shrimps collected from the abalone, *H. corrugata,* were permitted to choose between: Experiment 1—a test cell containing seawater only, Experiment 2—as Experiment 1 only with the transverse partition covered with an opaque screen to remove visual stimuli from the choice situation, Experiment 3—as Experiment 1 only with the abalone contained in a clear glass 4-liter beaker to remove chemical stimuli from the choice situation and Experiment 4—two control cells containing seawater only. Table II (Series 1) summarizes the results of these experiments. Significantly different choice between test and control cells was elicited only in the presence of chemical stimuli of host origin. Essentially the same number of shrimps located the host-containing cell when only chemical stimuli were present (Experiment 2) as when both visual and chemical were present, *e.g.*, the experimentally unaltered situation (Experiment 1).

To gain a fuller understanding of the results of Series 1 experiments, it was necessary to know if the presence of chemical stimuli triggered a response to current, since chemical stimuli were always presented in association with a directional flow of water emanating from the test and control cells. Table II (Series 2) summarizes the results of experiments designed to answer this question.

Shrimps in Series 2 experiments were collected from the host abalone, *H. rufescens*. Experiment 1 represents the experimentally unaltered choice situation. To determine if current alone had any effect, shrimps were permitted to discriminate between a test cell containing the model abalone and a seawater control cell both with (Experiment 2) and without (Experiment 3) a current in the apparatus. To eliminate current, the seawater inlets were closed. Substituting a model abalone for a live one in the test cell allowed presentation of visual stimuli without chemical stimuli while retaining the directional flow. The model consisted of a paraffin-filled abalone shell with 1.5 cm wide "epipodium" of black tape exposed beneath the ventral edge of the shell. As live abalone remained stationary when placed in the apparatus, a static model was judged an acceptable substitute. As can be seen, differential choice between test and control cells was not elicited in either experiment. Likewise, a test of association comparing the number of shrimps choosing the model-containing cell *vs.* the number not choosing it between the two experiments indicates no difference in the response of the shrimps $\chi^2 = 0.223$, $P = 0.50$–0.75). It appears that current itself does not effect the activity of the shrimps nor their response to visual stimuli.

To determine if the presence of non-directional chemical stimuli had any effect, shrimps were permitted to discriminate between test and control cells when non-directional chemical stimuli of host origin were present throughout the system, but in the absence of a flow (Experiment 4). Two specimens of *H. rufescens* (350 g), confined in a perforated plastic cup and swirled in the choice area of the apparatus for 1 minute prior to introduction of each shrimp, served to introduce non-directional chemical stimuli into the system. Assuming host effluents had an effective time stability of at least 6.0 minutes, host effluent was present in the choice area throughout the maximum time interval allowed for choice. This assumption, of course, could only be confirmed by a positive result, *i.e.,* by obtaining a significant change in response on the addition of such non-directional chemical stimuli. Differential choice was elicited in favor of the model-containing cell, suggesting that the presence of non-directional chemical stimuli may enhance the stimulus value of visual cues characterizing the model host. A test of association comparing the number of shrimps choosing the model-containing cell *vs.* the number not choosing it in this (Experiment 4) and in the control situation (Experiment 3—no current, no chemical), however, indicates that no significant increase in the level of activity occurred in the presence of the non-directional chemical stimuli ($\chi^2 = 0.178$, $P = 0.50$–0.75). It appears that non-directional chemical stimuli alone are not sufficient to affect the activity of the shrimps, although they may enhance directed activity in the presence of visual cues.

To determine if non-directional chemical stimuli serve to trigger a response to current, shrimps were permitted to discriminate between test and control cells when non-directional chemical stimuli of host origin were presented simultaneously with a directional flow (Experiment 5). No differential choice was elicited between the test and control cells. However, the method of introducing the chemical stimuli in this experiment should have dispersed host effluent throughout all compartments of the apparatus. Since a current was flowing under the transparent partition from both test and control cells, no difference should have existed in the stimulus pattern characterizing the two cells except for the visual stimuli of the model-containing cell. Experiments 2 and 3 indicate that visual stimuli with or without current elicit little activity. Thus, the combined number of shrimps reaching either cell can be considered as being most characteristic of the response to this stimulus situation. A test of association comparing the total number of shrimps choosing either cell *vs.* the number not choosing either cell in Experiment 5 and in the basic host response (Experiment 1—visual, current, and directed chemical stimuli of host origin), indicates no significant difference in the level of activity ($P > 0.995$). Host-oriented locomotion in the shrimp *B. harfordi* appears

TABLE II

Two-celled choice experiments: B. harfordi

Experiment	Contents of test cell	Stimulus modalities present*	Shrimps choosing		χ^2 Choice	P
			Test	Control		
Series 1						
1	*H. corrugata*	V, DC	26	1	23.0	<0.005
2	*H. corrugata*	DC	25	2	19.6	<0.005
3	*H. corrugata*	V	14	6	1.60	0.10 0.25
4	Seawater	—	4	4	—	—
Series 2						
1	*H. rufescens*	V, DC, C	26	0	26.0	<0.005
2	*H. rufescens*	V, C	2	2	—	—
3	*H. rufescens*	V	4	3	0.14	0.50–0.75
4	*H. ryfescens*	V, NDC	8	0	8.00	<0.005
5	*H. rufescens*	V, NDC, C	14	10	0.67	0.25–0.50
Series 3						
1	*H. cracherodii*	DC	25	0	25.0	<0.005
2	Seawater	-	5	2	1.29	0.25–0.50
3	*H. rufescens*	DC	24	0	24.0	<0.005
4	*H. corrugata*	DC	28	0	28.0	<0.005
5	*K. kelletia*	DC	8	8	—	—
6	*S. franciscanus*	DC	9	4	1.92	0.10–0.25
7	*M. crenulata*	DC	6	8	0.28	0.50–0.75
8	*I. caupo*	DC	7	2	2.78	0.05–0.10
9	*H. cracherodii*	DC	27	1	24.2	<0.005
10	Seawater	—	4	7	0.81	0.25–0.50

* V, visual; DC, directed chemical; NDC, non-directed chemical; C, current.

to result from the ability of chemical stimuli to release a response to directional water currents in these shrimp.

It was then asked: Is the distribution of the active substance(s) sufficiently restricted to explain the apparent specificity of association of the shrimps to molluscs of the genus *Haliotis?* Table II (Series 3) summarizes experiments extending over five consecutive days that permitted specimens of *B. harfordi* collected from the abalone, *H. cracherodii,* to discriminate between a test cell containing individuals of one of seven different species of test organisms and a control cell containing only seawater. An opaque white plastic screen placed over the transverse partition occluded visual stimuli from the choice situation. Test organism complements were equated to 350 ± 50 g wet weight. Significantly different choice was elicited by effluents of test organisms of the genus *Haliotis* (Experiments 1, 3, 4, 9). Differential choice was not elicited by effluents of two other gastropods, the neogastropod *Kelettia kelletii* (Experiment 5) and the archeogastropod *Megathura crenulata* (Experiment 7). Similarly, differential choice was not elicited by effluents of the echiuroid *Urechis caupo* (Experiment 8) nor of the echinoid *Strongylocentrotus franciscanus* (Experiment 6), both reported to be hosts of congeneric *Betaeus* species (Hart, 1964). Agreement of initial and terminal replicates of the basic host response (Experiments 1, 9) suggests the lack of response in the latter experiments was not the result of a temporal change in responsiveness of the shrimps.

The total number of shrimps locating tests cells containing *Haliotis* spp. was greater than for the non haliotid species. A test of association on the results of the most and least extreme distributions obtained with *Haliotis* effluents (Experiments 3, 4) indicates no significant difference between the numbers of shrimps locating the test cell in these experiments ($\chi^2 - 0.935$, $P - 0.25$–0.50). A test of association on the results of the least extreme distribution obtained with a *Haliotis* effluent (Experiment 3) and the least extreme distribution obtained with non-host effluent (Experiment 6), however, indicated a significant difference between the number of shrimps locating the test cells ($\chi^2 - 22.9$, $P - < 0.005$). It follows that the remaining and more extreme distributions obtained to non-host effluents are also significantly different from the distribution obtained in Experiment 3.

The Betaeus-Strongylocentrotus *association*

Experiments utilizing arena apparatus. The question was first asked whether shrimps in association with the urchin *S. franciscanus* require both chemical and

TABLE III

Arena experiments: B. macginitieae — S. franciscanus vs. D. imbricata

Experiment	Stimulus modality removed	Shrimps tested	Shrimps choosing		χ^2 choice	P
			Host	Control		
1	None	111	89	13	56.6	<0.005
2	Chemical	111	63	25	15.4	<0.005
3	Visual	96	63	12	35.4	<0.005
4	None, hosts in all corners	99	44	47	0.09	0.75–0.90

visual stimuli of host origin to effect host location. Shrimps were permitted to make a choice when presented with two corner compartments containing host urchins and two containing the control organism. Visual or chemical stimuli were then selectively removed from the choice situation. Table III summarizes the results of these experiments which extended over a period of 8 days. Experiment 1 represents the basic host-location response of shrimps in the arena apparatus when neither chemical nor visual stimuli were altered, *i.e.,* the "natural" stimulus condition. A significantly greater number of shrimps selected the two host-containing cells. When both the test and control organisms were contained in clear glass 4-liter beakers to eliminate chemical stimuli from the choice situation and an otherwise identical experimental protocol maintained (Experiment 2), significantly more shrimps still selected the host-containing compartments. Likewise, with the corner compartments covered with eight layers of white cheesecloth so as to exclude visual stimuli yet not alter the flow characteristics of the system and retain chemical stimuli (Experiment 3), significantly more shrimps selected the host-containing compartments. Experiment 4, in which host sea urchins were substituted for the seastars in the control compartments (*i.e.,* all compartments contained host urchins) suggests that final distributions of this series of experiments were not biased by the experimental procedure itself.

Experiments utilizing 2-celled choice apparatus. For the reasons previously described, more detailed analyses utilized the 2-celled choice apparatus. Repetition of the above described experiments provided a basis for comparison of the two techniques of behavioral quantification. Table IV (Series 1) summarizes the results of these experiments. Shrimps were permitted to select between a test cell containing the host urchin and a control cell containing seawater only. In the basic stimulus situation, where both chemical and visual stimuli were experimentally unaltered, significantly more shrimps selected the host-containing cell (Experiment 1). With the host urchin contained in a clear glass 4-liter beaker placed in the test cell and a seawater filled beaker placed in the control cell, significantly more shrimps still selected the host-containing cell (Experiment 2). With an opaque white plastic screen attached to the transverse partition thus masking visual stimuli from the choice situation while not interfering with chemical stimuli, significantly more shrimps again selected the host-containing cell (Experiment 3). Neither stimulus modality acting alone, however, elicited host location to the extent that both did when presented together. Very few shrimps made a choice in the absence of any stimuli of host origin (Experiment 4). These data are in agreement with those of the arena experiments indicating that either chemical or visual stimuli of host origin are sufficient to effect host location by these shrimps.

The question was then asked to what extent can the response to chemical stimuli explain the apparent specificity of the *B. macginitieae-Strongylocentrotus* association. Screening the transparent plexiglass divider with a thin sheet of opaque white plastic arranged so as not to alter the flow characteristics of the apparatus effectively blocked visual communication between the cells and the choice area, while allowing free passage of chemical cues. The mean weight of the test organisms utilized in each experiment was 200 ± 25 g. In a series of experiments extending over five consecutive days, shrimps were permitted to

Table IV

Two-celled choice experiments: B. macginitieae

Experiment	Contents of test cell	Stimulus modalities present*	Shrimps choosing		χ^2 Choice	P
			Test	Control		
Series 1						
1	*S. franciscanus*	V, C	28	1	25.0	<0.005
2	*S. franciscanus*	V	22	0	22.0	<0.005
3	*S. franciscanus*	C	22	1	19.2	<0.005
4	Seawater	—	2	2	–	
Series 2						
1	*S. franciscanus*	C	25	0	25.0	<0.005
2	Seawater	—	4	2	0.66	0.25–0.50
3	*S. purpuratus*	C	20	0	20.0	<0.005
4	*L. anamesus*	C	5	1	2.66	0.10–0.25
5	*S. parvimensis*	C	8	6	0.57	0.25–0.50
6	*D. imbricata*	C	8	6	0.57	0.25–0.50
7	*H. rufescens*	C	9	6	0.60	0.25–0.50
8	*U. caupo*	C	8	5	0.69	0.25–0.50
9	*S. franciscanus*	C	21	1	18.2	<0.005
10	Seawater	—	3	5	0.50	0.50–0.75

* V, visual; C, chemical; —, neither visual or chemical.

discriminate between a test cell containing one of seven different species of test organism and a control cell containing seawater only (Table IV, Series 2). Only effluent of the congeneric echinoids, *S. franciscanus*, the natural host (Experiments 1, 9), and *S. purpuratus*, (Experiment 3), elicited differential choice between test and control cells. Differential choice was not elicited by effluents of the non-host echinoid, *Lytechinus anamesus* (Experiment 4), nor the non-echinoid echinoderms, *Stichopus parvimensis* (Experiment 5) and *Dermasterias imbricata* (Experiment 6). Likewise, effluents of the abalone, *Haliotis rufescens* (Experiment 7), and the echiurid worm, *Urechis caupo*, (Experiment 8) both reported hosts for congeneric *Betaeus* species (Hart, 1964), failed to elicit differential choice. A test of association indicates that the final distribution elicited by effluents of the urchin, *S. purpuratus* (Experiment 3), does not differ significantly from the more extreme of the two distributions elicited by effluents of the natural host (Experiment 1) ($\chi^2 = 1.46$, $P = 0.1–0.25$). That the distribution elicited by non-strongyloid effluents differs significantly from that obtained in Experiments 1, 3, and 9, is indicated by a test of association of the least extreme distribution obtained with *S. franciscanus* effluent (Experiment 9) and the least extreme distribution obtained with a non-host effluent, that of the abalone, *H. rufescens* (Experiment 7) ($\chi^2 = 13.3$, $P = < 0.005$). Only a few individuals responded in the absence of any stimuli of host origin (Experiments 2, 10). Agreement of initial and final repetitions of the basic host response (Experiments 1, 9) support the hypothesis that no change occurred in the response level of the shrimps during the duration of the experimenal period.

The question was then asked to what extent can the response to visual stimuli of host origin explain the apparent specificity of the *B. macginitieae-Stronglyocentrotus* association. These experiments utilized the static modification of the 2-celled choice apparatus in which chemical stimuli and the carrier flow are absent from the choice situation. As a preliminary experiment, shrimps were permitted to select between a cell containing the host *S. franciscanus* and a cell containing one of five different test organisms, the host, *S. franciscanus,* the abalone, *Haliotis rufescens,* the holothuroid, *Stichopus parvimensis,* the alternate host, *Strongylocentrotus purpuratus,* and the giant keyhole limpet, *Megathura crenulata.* These animals represent the predominant, non-sessile, macrobenthic fauna of the Santa Barbara collection site. All test organisms were equated for displacement volume (+ 50 ml). Shrimps selected the host-containing cell in all cases except in the pairing of the limpet, *Megathura,* and the host urchin. These experiments were not pursued further, however, due to the difficulty in equating such diverse organisms for "unit" characteristics.

As an alternative approach to gaining an understanding of the visual basis of the shrimp-urchin association, an effort was made to determine which component(s) of the total visual pattern characterizing *S. franciscanus* is (are) utilized by the shrimps to effect visually-mediated host recognition. These experiments quantified the ability of shrimps to visually distinguish between two simultaneously presented objects (Cell A and Cell B). Any shrimp not moving away from the release point by 5.5 minutes was touched on the telson with a camel's hair brush, which served as sufficient stimulus to initiate movement to criterion in

TABLE V

B. macginitieae: *analysis of stimulus parameters visually characterizing* Strongylocentrotus franciscanus

Experiment	Cell A	Shrimps choosing A	Shrimps choosing B	Cell B	χ^2 choice	P
1	Urchin (9.0)*	23	7	Urchin (6.4)	8.52	<0.005
	Urchin (6.4)	21	9	Urchin (4.5)	4.80	0.025–0.05
2	Spineless urchin (8.0)	18	12	Intact urchin (4.0)	1.20	0.25–0.50
3	Disk (10.0)	14	16	Urchin (7.5)	1.34	0.75–0.90
	Disk (10.0)	18	12	Urchin (5.0)	1.20	0.25–0.50
	Disk (10.0)	28	2	Urchin (3.5)	22.4	<0.005
4	Disk (9.0)	27	3	Patterned disk (9.0)	19.2	<0.005
5	Black disk, white bkgd. (3.8)	24	5	White disk, black bkgd. (3.8)	12.5	<0.005
6	Disk (9.0)	20	9	Serrated disk (9.0)	4.16	0.025–0.05
	Disk (9.0)	17	13	Square (7.9)**	0.53	0.25–0.50
	Disk (9.0)	7	23	Square (8.9)	19.2	<0.005
	Disk (6.0)	15	14	Square (5.3)	0.03	0.75–0.90
	Disk (9.0)	14	15	Triangle (12.5)	0.03	0.75–0.90
	Disk (9.0)	15	13	Inverted triangle (12.5)	0.14	0.50–0.75

* ( ) indicates maximum test diameter or diameter of disk model, cm.
** ( ) indicates length of single side of model, cm.

most shrimps within the 6.0 minute test period. Table V summarizes the results of these investigations.

To determine whether urchins are discriminated by size, shrimps were permitted to choose between a moderate and a larger-sized urchin, as well as between the same moderate-sized urchin and a smaller one (Experiment 1). In each case, the cell containing the larger urchin of the pair was favored. To test the possibility that larger shrimps preferentially choose larger urchins, a two-way contingency analysis was applied to the number of small (< 1.5 cm total length) and large (> 2.5 cm total length) shrimps choosing the larger of the two hosts in each pairing. Moderate sized shrimps were not included in this calculation in order to produce more discrete size classes of small and large individuals. For both pairings, the hypothesis of no difference in response between small and large sized shrimps could not be rejected ($\chi^2 - 1.82, 1.56$; $P - 0.10$–0.25).

To determine if urchins are recognized by the presence of spines, shrimps were permitted to choose between a large urchin (8.0 cm test diameter) from which all spines had been clipped off to within 0.5 cm of the test and a small urchin (4.0 cm test diameter) from which only the tips of the longest spines had been clipped to obtain a peripheral diameter of 9.0 cm (Experiment 2). Both "urchins" had the same peripheral diameter thus minimizing experimental bias due to a size difference between the two test objects. If the presence of spines or the spinose form was an attractive parameter, shrimps should favor the smaller of the two urchins, *i.e.,* the one with spines essentially intact. Neither cell was favored, however. If spines are not necessary for urchin recognition, it should further be possible to construct a solid dark colored model that could not be differentiated from an intact urchin of equal effective visual diameter. Two-dimensional models fashioned from thin sheet plastic and painted flat black were presented by mounting them on a panel of clear plastic centered vertically in the test cell. Since it was not possible *a priori* to equate disk diameter with urchin peripheral diameter, a series of three pairings was conducted in which shrimps were permitted to choose between a 10 cm diameter black disk and one of three different sized urchins (Experiment 3). No discrimination was obtained in favor of either cell when intact urchins of 7.5 and 5.0 cm test diameter were paired with the disk. With a more extreme size differential, a 10 cm disk *vs.* a 3.5 cm test diameter urchin, however, the cell containing the solid disk was favored.

A third experiment was directed towards evaluating spines as a parameter of recognition. Shrimps were permitted to choose between a 9.0 cm diameter solid black disk and a 9.0 cm diameter patterned black-white disk consisting of a "checker-board" of alternating black and white squares 1.0 cm on a side (Experiment 4). If shrimps recognize the spinose form of the urchin on the basis of the internal contrast, the patterned black-white disk, with its greater internal contrast, should be favored over the solid disk of equal peripheral diameter. The cell containing the solid disk was favored over that containing the patterned disk.

To determine if urchins are differentiated as a discrete form *per se* or as a contrasting pattern with the background, the transparent "windows" of the test cells were masked with opaque screens. The size of a disk was calculated so that its total area equaled one-half the area of the opaque screens and a disk centered on each screen. Shrimps were permitted to distinguish between a white disk presented against a black background and black disk presented against a white background (Experiment 5). Both models contain equal areas of black and white. Both contain equal zones of black-white boundary. Both models should be equally attractive if shrimps are responding to the amount of light-dark contrast. The cell containing the black disk against the white background was favored over the reverse combination.

To determine if urchins are differentiated on the basis of their peripheral outline, shrimps were permitted to choose between a 9.0 cm diameter disk and a series of two dimensional shapes (Experiment 6). A 9.0 cm diameter solid black disk was favored over a 9.0 cm diameter serrated black disk with 28 equally-spaced serrations cut radially to a depth of 2.0 cm. Neither cell was favored on presentation of a solid, black square, 7.9 cm on a side, and a 9.0 cm diameter solid disk, these models enclosing equal areas. However, if the size of the square shape was increased so that its area equaled 1.3 times that of the 9.0 cm diameter disk (*i.e.,* 8.9 cm per side), the shrimps favored the cell containing the square shape. To control the possibility that the overall size of the models exceeded the visual angle subtended by the shrimps' eyes, the square-disk pairing was again presented except that the area was reduced and equated to that of a 6.0 cm diameter disk (square 5.3 cm per side). As with the larger models, neither cell was favored in this latter pairing. Finally, shrimps did not discriminate between 9.0 cm diameter solid black disk and a solid black equilateral triangle of equal area (12.5 cm per side), either with the triangle oriented point upward or inverted with the point downward. Experiment 6 supports the conclusion that visual recognition of the host urchin, *S. franciscanus,* by the shrimps is not based on the perception of the urchin as a round or circular form.

Discussion

The data support the hypothesis that *B. harfordi* is able to effect distant host recognition utilizing chemical stimuli of host origin alone. It is not suggested that visual cues are entirely without effect when available. Indeed, Experiments 1–3 and 2–4 (Table II) suggest that visual stimuli of host origin may elicit a low response under certain conditions. However, both these apparent responses to visual stimuli can also be explained as a generalized response toward the only contrasting object in an otherwise monotonous choice situation. The results of Experiment 2–2 (Table II) which incorporate essentially the same stimulus parameters as Experiment 1–3 (Table II) did not indicate any tendency of the shrimp to select the model-containing cell. It is interesting to note that subtidal species of *Haliotis* are frequently heavily encrusted with epiphytic growths and contrast little with the surrounding substrate (Cox, 1962). However, the black epipodium of at least one of the subtidal species, *H. rufescens,* could offer sufficient contrast with the background to facilitate visual recognition at close range.

A question arises as to the ability of chemical stimuli alone to effect directed locomotion towards an odor source (Fraenkel and Gunn, 1961; Gage, 1966). In the marine benthos local water turbulence and surging would disrupt diffusion gradients required for chemotaxic orientation. It appears that *B. harfordi* can [illegible] the directional component of a carrier current containing host factor to effect host location by moving "upstream" in the presence of the appropriate chemical releaser. This mechanism reportedly occurs in other crustaceans (Allee, 1916; Luther, 1930), although to the authors' knowledge it has not been investigated in any detail. Laverack (1962) has demonstrated low frequency tactile receptors in *Homarus* chelae, sensitive to water currents down to 0.3 cm/sec. Low threshold chemoreceptors have been well documented in the crustacea (*e.g.*, review of Laverack, 1968). Such a mechanism would be adaptive in that it would not require chemoreceptor competence sufficient to discriminate the very small increments in chemical concentration required for chemotaxic orientation, but merely the presence or absence of the attractant.

Specificity experiments suggest that *B. harfordi* discriminates a chemical substance or complex of substances containing sufficient information for recognizing gastropods of the genus *Haliotis* from other gastropods and from the non-molluscan hosts of other betaeid shrimps. The data do not eliminate the possibility that this chemically-mediated genus-specific recognition is based on quantitative rather than qualitative differences in the attractants. The fact that equal masses of test organisms were contained directly in a continuously flowing seawater wash minimizes experimentally induced variations in stimulus concentration, so even quantitative differences in the same attractant must be considered as potentially significant mechanism for maintenance of this association. Recently, evidence has been presented that quantitative odor differences are at least partially responsible for mediating escape attack behavior in a marine gastropod (Snyder and Snyder, 1971).

Previous investigations indicate a relative high degree of chemosensory competence in crustaceans. Symbiotic pinnotherids, *Pinnixa chaetopterana*, discriminate effluents of host polychaetes of the genera *Chaetopterus* and *Amphritrite* from those of the non-host polychaete genera, *Nereis* and *Arenicola* (Davenport *et al.*, 1960). Another symbiotic pinnotherid, *Dissodactylus mellitae*, discriminates its host echinoid, *Mellita quinquiesperforata*, from six other species of echinoderms, although it reportedly can be conditioned to respond to another flattened echinoid, *Encope mitchelini* (Gray *et al.*, 1968). More specific discrimination has been reported (Carton, 1968) for the parasitic copepod *Sabelliphilus sarsi* which can discriminate by chemical means between its host polychaete, *Spirographis spallanzani* and two non-host but congeneric polychaetes, *S. pavonina* and *S. spallanzani* var. *brevispira*. By nature of their action, crustacean sex phermones (Atema and Engstrom, 1971; Kittredge, Terry and Takahashi, 1971; Ryan, 1966) could also be considered species-specific chemical attractants, but the possibility that other stimulus modalities confer the species specificity to crustacean chemically-mediated mate recognition remains to be disproven.

B. macginitieae, in contrast to *B. harfordi*, appears to use both visually and chemically mediated information for distant host recognition. The interaction of chemical and current stimuli was not adequately investigated for *B. macginitieae*, and thus is not reported here. Visually mediated information would contain sufficient directionality, however, to allow directed locomotion in situations offering both chemical and visual stimuli, even in the absence of current flow. It is possible that chemical and visual cues, acting together enhance the value of the stimulus situation to the shrimps. More shrimps located the host urchins in the arena experiment presenting both stimuli simultaneously than in those lacking either visual or chemical cues (Table III, Experiments 1, 2, 3). Such apparent enchancement does not necessarily result from neural summation of the sensory information contained in the two stimulus modalities, however, since it could also be explained by chemically released hyperactivity increasing the probability that visually directed locomotion towards the host occurs within the test period. The data do not allow resolution of this question, but the phenomenon is worthy of further investigation. Symons (1964) earlier reported that the number of feeding movements nearly doubled in the crab *Hemigrapsus oregonensis* when elicited by both chemical and tactile stimuli together than by either stimulus modality operating alone.

The specificity of the *Betaeus-Strongylocentrotus* association in nature is somewhat unclear. Hart (1964) described the species with nine specimens, one pair collected from *S. purpuratus,* one female from *S. franciscanus,* and six with no host record. In an area abundant with both urchin species, over 1000 specimens of *B. macginitieae* were collected, associated in all but one instance with specimens of *S. franciscanus.* This fact, along with the fact that *B. macginitieae* is homochromous with *S. franciscanus* and behaviorally adapted to move among the long spines of this urchin (Ache, 1970) suggests that *S. franciscanus* may be the "preferred" host of *B. macginitieae* and *S. purpuratus* a secondary host.

Chemical stimuli contain sufficient information to allow the urchin symbionts to discriminate urchins of the genus *Strongylocentrotus* from other echinoderms and from the non-echinoderm hosts of other betaeid shrimps, a level of sensory competence at least functionally similar to that of *B. harfordi.* Visually mediated information is sufficient to allow *B. macginitieae* to further discriminate between the two reported strongylocentrotid hosts. The long-spined, brick red (occasionally to light red) *S. franciscanus* is morphologically distinct from the short-spined, smaller, light purple *S. purpuratus* (Ricketts and Calvin, 1968). Thus *B. macginitieae* with its demonstrated ability to visually discriminate large, dark "solid" objects could differentiate between the two urchin species. Visually-mediated behavior is not commonly reported to occur in aquatic crustaceans, although its role is rather well documented in the control of sexual and agonistic behavior of semi-terrestrial species (*e.g.,* reviews of Schone, 1968; Salmon and Atsaides, 1968; Wright, 1968). Alverdes (1930) noted that the aquatic branchyuran *Carcinus maenas* and the anomuran *Eupagurus bernhardus* confronted by two black screens will move between them, but before doing so beat their antennae in the direction of each of the screens, behavior he interpreted as suggesting that perception of the screen as objects does occur. Visually-mediated food location behavior has been reported for the aquatic anomuran (*Clibanarius vittatus* (Hazelett, 1968) and the intertidal brachyuran *Pachygrapsus crassipes* (Hiatt, 1948) although these latter observations were conducted on crabs in air. Symons (1964) was unable to demonstrate either a releasing or directing effect of visually-mediated stimuli on the feeding behavior of the aquatic brachyuran *Hemigrapsus oregonensis.*

Question arises as to the ability of visual stimuli acting alone to effect a response specific to *S. franciscanus* if *B. macginitieae* is not responding to any visual parameter uniquely characteristic of its host. Experiments showed that the predominately black hemispherical *Megathura* was not distinguished from the host *S. franciscanus* in a paired choice situation, although visual stimuli proved sufficient for discrimination of the host urchin from other lighter-pigmented organisms. The possibility must be considered that few other large, dark-pigmented organisms like *Megathura* may occur in the subtidal rocky habitat of the range ascribed by Hart for the *Betaeus-Strongylocentrotus* association (Santa Catalina Island, Monterey, California). Further experimentation, however, is necessary to clarify this point.

A visual receptor capable of rudimentary form vision would be sufficient to effect the visually-mediated behavior demonstrated by the shrimps. As noted by Carthy (1958), what appears to be simple form recognition of dark shapes can frequently be explained by the alternative hypothesis of a negative phototaxis towards a zone of reduced light intensity. That a more complex response than simple negative phototaxis is involved is demonstrated by the preference of *B. macginitieae* for the black circle presented against a white background over the white circle presented against a black background (Table V, Experiment 5). Both models presented equal zones of contrast and equal areas of reduced intensity. This is not to imply that the shrimps are not negatively phototactic; it has been shown they are (Ache, 1970). The peripheral outline of the model does not appear to be an active parameter in discrimination (Table V, Experiment 6) suggesting that the attractive factor may be more the "solidness" of the form than its specific shape—*e.g.*, circular or semi-elliptical as the urchin test. The preference of shrimps for the solid circle over the black-white checkerboard-patterned model (Table V, Experiment 4) also supports this idea.

The possibility of color discrimination has not been eliminated by these experiments. Two factors tend to discredit the possibility that *S. franciscanus* is recognized on the basis of color. Black models proved as equally attractive as naturally pigmented urchins, when equated for effective visual diameter (Table V, Experiment 3). Also the extinction coefficients of coastal seawaters are greater for longer wavelengths of visible light required for color discrimination of a red pigmented organism (for coastal water off Southern California-Young and Gordon, 1939). However, Wald and Seldin (1968) have demonstrated differential sensitivity of two components of the ERG in the shrimp *Palaemontes vulgaris* which they suggest may represent the red- and violet-sensitive components of a visual mechanism for color differentiation. The results of the present experiments, however, indicate that intensity discrimination would be sufficient to explain the visually-mediated component of host recognition.

It appears then that information from several sensory modalities is utilized by both *B. harfordi* and *B. macginitieae* to effect their respective symbiotic relationships. This mechanism serves to reduce the demands on the competence of any one receptor type, while maximizing the discriminating ability of the shrimps both in regards to stimulus directionality (the *B. harfordi* studies) and stimulus specificity (the *B. macginitieae* studies). The present experiments do not allow resolution of whether the action of such multi-modal information is simply additive or involves summation and perhaps additional integration in higher neural centers. Certainly, centers of higher order neural integration receiving visual information and input from other sensory modalities, including chemosensory information, are known to exist in the eyestalks of *Panulirus argus* (Maynard and Dingle, 1963; Maynard and Yager, 1968) and probably in other decapod species

(Hazlett, 1971). Work towards resloving this question is currently in progress using crustaceans of several species.

Summary

1. The sensory basis of host-oriented locomotion in the caridean *Betaeus macginitieae* contrasts with that of the congeneric *B. harfordi*. Both of these shrimps can locate their respective host organisms utilizing chemical stimuli of host origin. Only *B. macginitieae* demonstrates the ability to utilize visual stimuli for this same purpose.

2. By using information contained in multiple stimulus modalities, *B. macginitieae* is able to maintain a more restricted host association than its congener.

3. Visual recognition of its urchin host by *B. macginitieae* does not involve any parameter of the total visual pattern of the urchin that uniquely characterizes the urchin species, but appears to be a generalized response to larger, dark forms of undefined peripheral outline.

4. Positive rheotaxis in the presence of appropriate non-directional chemical stimuli is suggested as the mechanism by which *B. harfordi* effects chemically-mediated host location.

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VISUAL ORIENTATION AT THE WATER SURFACE BY THE TELEOST *ZENARCHOPTERUS*[1]

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In seeking quantitative documentation that certain teleosts can perceive the *e*-vector of linearly polarized light (earlier work reviewed in Waterman, 1972), a number of field experiments have been carried out on the viviparous tropical halfbeak *Zenarchopterus* (Hemirhamphidae). Most of these were underwater studies conducted with single fish enclosed in a covered transparent vessel and exposed to various illumination patterns including both natural and imposed polarized light (Waterman and Forward, 1970; 1972).

Although halfbeaks were behaviorally responsive in this submerged situation, they normally swim at the water surface. In order to include this potentially important feature of the fish's visual and tactile environment, additional experiments were conducted on land with the fish swimming in an open experimental vessel. Under such conditions a fairly vigorous basic polarotaxis was evoked that was somewhat different from that previously observed. Also new evidence was found relating to other components in the fish's visual orientation. These studies of *Zenarchopterus'* visually evoked directional behavior at the water-air interface are the subject of the present report.

To begin with some precise definitions of terms describing animal orientation to light may be helpful. *Phototaxis* is the directionally oriented response of an organism to light intensity patterns (Kuhn, 1919; Fraenkel and Gunn, 1940; Jander, 1970). The comparable response to light polarization patterns is *polarotaxis* (Waterman, 1966), *i.e.*, a directional response in relation to a given plane or pattern of polarization. When the resulting orientation (body axis alignment or steering direction of locomotion) has a fixed, non-graded angular relation to the stimulus, *e.g.*, heading toward the light (0°) or away from it (180°) (phototaxis), or at 0°, 45°, 90°, 135° to the *e*-vector (polarotaxis), then it may also be called a "basic" response or *basitaxis* (= "basotaxis" of Jander, 1963a, 1963b).

In contrast to basitaxis there is another relatively simple type of response to spatial differences in light intensity. This is *photomenotaxis*, or the light compass reaction (von Buddenbrock, 1917), in which orientation may be at *any* temporarily fixed angle to the stimulus source. A comparable compass orientation depending on linearly polarized light is also well known in many arthropods (Waterman, 1966).

For behaviorally significant direction-finding which utilizes celestial cues, a menotaxis rather than a basitaxis would ordinarily be needed, since the course

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[1] This research was supported by grants from the U. S. Public Health Service, the National Geographic Society and the National Aeronautics and Space Administration.

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heading required would depend on the animal's present position relative to the location of its "goal." Thus as the animal moves the angular relationship between the compass direction connecting these two points and the reference cue will usually vary with time. Moreover, if the orientation is to be accurately maintained by celestial reference for more than a few minutes, the compass reaction would have to be time compensated to allow for the apparent movement of the sky and celestial bodies as the earth rotates (von Frisch, 1950; Kramer, 1950).

Fishes have been shown to be capable of a *time-compensated sun compass orientation* (reviews: Hasler, 1966; Harden Jones, 1968; Waterman, 1972). This ability implies that from their underwater vantage point fishes are able to determine the sun's azimuth direction and possibly its altitude. Close to the surface and in a flat calm this might be accomplished by direct observation of the sun's disc or sky polarization. Otherwise it could be derived either from the radiance distribution in the water or from the underwater polarization pattern, both of which are directly dependent on the sun's position (Waterman, 1954; Jerlov, 1968; Lundgren, 1971).

The light intensity distribution is probably suitable for accurate localization (within say 6°) in shallow water (5–10 m). However, the useful information from this radiance pattern decreases rapidly with increasing depth (Harden Jones, 1968). A more precise indicator of the sun's position which penetrates to greater depths is the polarization pattern underwater (Waterman, 1955; Ivanoff and Waterman, 1958). Therefore, demonstration of polarotaxis by fish would imply that they are capable of perceiving this component of natural submarine illumination. In turn this suggests that they could use the underwater polarization pattern to localize the sun.

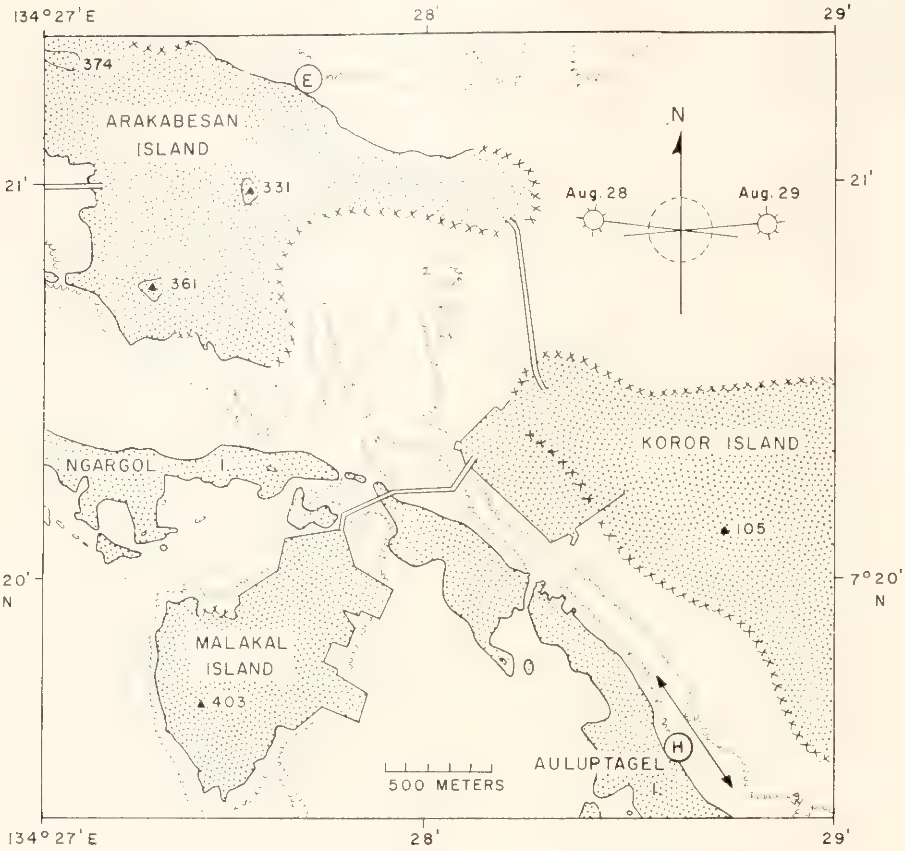
We present herewith new evidence for polarotaxis as well as for time compensated sun compass orientation by the fish *Zenarochopterus.*

EXPERIMENTAL METHODS

The experiments were carried out in Palau, Western Caroline Islands (U. S. Trust Territory of the Pacific Islands) on August 28th and 29th, 1970. The general methods employed resemble those for the corresponding underwater studies (Waterman and Forward, 1972) except that the fish were tested in a transparent vessel open to the air with a free water surface.

The experimental site was the broad cement apron of an abandoned seaplane ramp on the north shore of Arakabesan Island (*E,* Fig. 1). Single fish were placed in a cylindrical transparent plastic container, 19 cm in diameter and 7.5 cm deep. The vessel was positioned above an intervalometer-controlled Robot camera, and screened laterally and downward by white, cylindrical screens and a diaphragm. Thus, the fish had a free view of the sky through the water surface, but was prevented from seeing surrounding landmarks, the experimenters or the equipment (except for the camera lens). To minimize reflection from the meniscus or from the cylinder's plastic wall in air, the vessel was carefully filled with seawater just to its brim. Freshly caught, experimentally naive juvenile *Zenarchopterus dispar* (Cuvier and Valenciennes) 40–50 mm in length were used. All fish were collected at a localized site on Auluptagel Island (*H,* Fig. 1).

During the experimental period, the sun's bearing and elevation were deter-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134°27'E
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FIGURE 1. Map of Arakabesan, Auluptagel, and nearby area of the Palau Islands. The experimental site was at *E* and all the *Zenarchopterus* used were collected at *H*. The double headed arrow near the latter indicates the main trend of the inter-island channel adjacent to *H* (channel axis 150–330°). Island peak elevations are given in feet. Outer edge of coral reefs are represented by scalloped outlines, mangrove areas by cross-hatching.

mined every 10 min with compass and sextant (Fig. 2). To document cloud cover conditions, photographs of the sky were taken at similar intervals with a camera having a fisheye lens. The sky generally was 35–40% obscured by light scattered clouds on the two days concerned. However, fish orientation was recorded only when the sun was clearly visible. When a small cloud briefly obscured the sun, measurements were suspended until the sun was again shining.

Twenty-four specimens of *Zenarchopterus* were studied, each under four optical conditions: (1) without the polarizer (NF condition), during which the fish was exposed only to the natural illumination of sun and sky; and (2, 3 and 4) with a Polaroid linear polarizer (Type KN36) placed over the experimental vessel (WP condition) and oriented at three different directions with respect to the sun's bearing (Fig. 3). Since six planes of polarization were tested altogether (0–180°, 30–210°, 60–240°, 90–270°, 120–300°, 150–330°), only half were used with an individual fish.

For each condition, 10 consecutive photographs of the fish's headings were made at 10 sec intervals timed and counted by the intervalometer. The filter was then changed manually to its next position or removed for the NF condition. After a 10 sec pause to let the fish settle down, another 10 frame sequence was made, etc. The order of presentation of the conditions, as well as the angle of the polarizer were randomly selected with the constraints that no condition was used twice with a given fish, and all conditions were tested an equal number of times.

The angle between the fish's longitudinal axis and the sun's bearing taken as 0° reference was measured to the nearest 10° from the photographs. For analysis these headings were grouped into 30° sectors centered to coincide with the six polarization planes tested. Obviously the positions and angular extent of these sectors are fixed and the response variable is the number or percentage of observed orientations which falls within each.

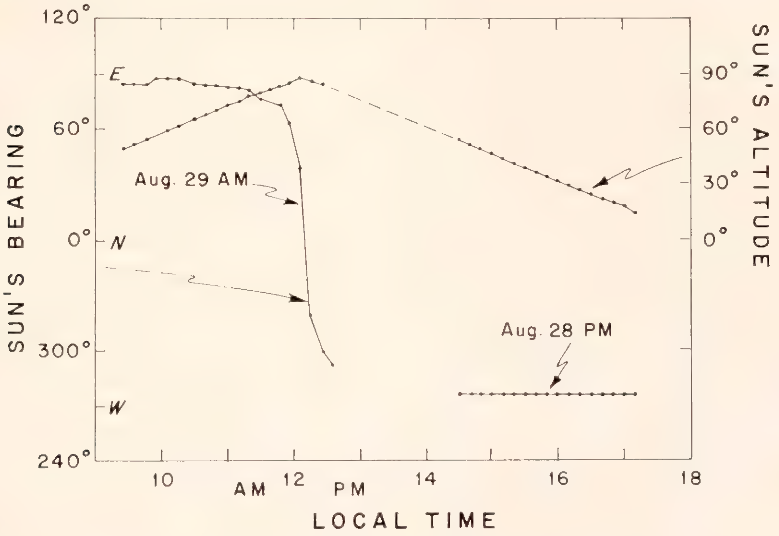

FIGURE 2. Sun's bearing and altitude during the experimental periods. Note that for August 28th the solar bearing was constant near W by WNW during the full period. On the 29th the bearing was stable for the first 2.5 hours, then it shifted very rapidly. For this reason measurements made during the last 30 min (4 of the 12 fish) were excluded from the main analysis.

The experimental design permits the overall data to be analyzed in several ways to test for various significant stimulus components. Thus the total distribution as recorded has the sun's bearing at 0° and the imposed *e*-vector randomized (Fig. 3). When transposed to geographical coordinates (0° = North) the *e*-vector is still at random but the sun's bearing appears mainly in two opposite directions. Their effect can be determined by separating the data for days.

The NF data can be compared with the randomized WP distribution to check for possible behavioral changes due to other features of the polarizer than its *e*-

vector orientation. Then the WP counts can be transposed so that all the imposed *e*-vectors are aligned with the 0°–180° plotting axis. This distributes the influence of the sun and any geographically derived effects leaving just polarotaxis as the predominant source of orientation in the resulting pattern. Finally the NF data can be compared with those for any particular imposed *e*-vector orientation.

To begin with the responses of the 24 individual fishes were studied in detail. Previous experience showed that "inattentive" fish significantly decrease the overall response level to potential orienting stimuli (Waterman and Forward, 1972). Obviously a completely inactive fish and one orienting consistently "on the beam" will both maintain constant headings. However, the inattentive animal can be recognized by its failure to change headings over a long period even when the experimental conditions are altered. Inattention may also appear as a continuously changing orientation at a constant or high velocity. In such cases no externally cued directional preferences may be involved.

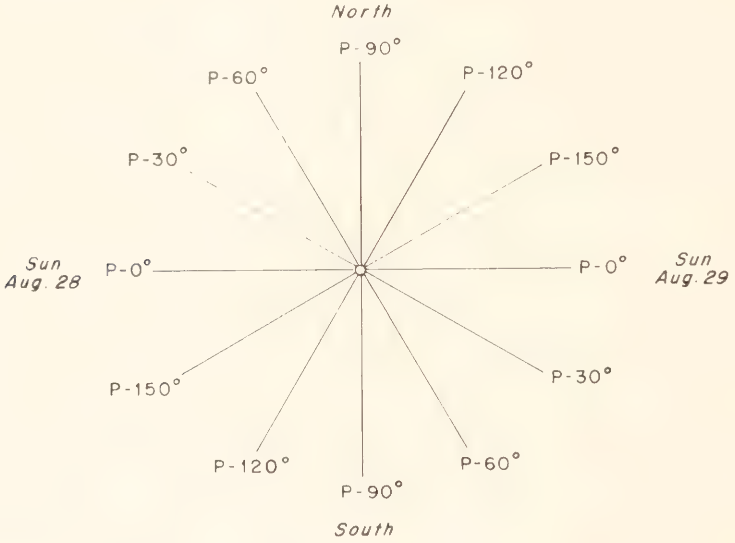

FIGURE 3. Angular relationship between North, sun's azimuth and polarizer *e*-vector directions (P-0°, *etc.*).

The major results presented below are derived from data selected to minimize these difficulties. However, the effects of such selection have been repeatedly checked by comparisons with the total data and with distributions resulting from alternative selective procedures. For example the last four fish run on August 29 were eliminated from prime consideration because the sun was changing its bearing very rapidly during their runs (Fig. 2). Also at that time the solar zenith distance, and hence the sun's potential influence in determining azimuth, was minimal as was the sky polarization near the zenith.

Indeed these fish showed different but less coherent orientation preferences than those run with steady sun's bearings. Nevertheless addition of their counts to those of the other 18 orienting fish does not alter the location of significant sectors relative to the North, sun, or *e*-vector. Nor do they significantly affect the overall NF distribution.

Furthermore two other fish from the afternoon sequence were eliminated from the selected data because they scarcely changed their headings throughout the set of four conditions tested for each. Hence 18 of the total 24 fish provide the orientation headings analyzed in most detail.

Moreover for all fish rather large angular changes of direction sometimes did occur between frames. To decrease the influence of such more rapid turning, we have for our selected data rejected orientation measurements which differed from their predecessor by more than 20°. The remaining headings are interpreted as "pauses" in directions preferred by the fish and possibly determined by some external clue for azimuth.

Since behavior patterns of the individual fish were different the number of pauses observed under each experimental condition varied. Therefore, to prevent over or under representation by any one condition when totaling the results, the data for each condition were converted into percentage responses in each 30° sector. Where different conditions are combined, the percentage responses in each angular category are totaled and divided by the number of conditions used.

Ninety-nine per cent binomial confidence limits were computed for the observed frequency of responses in each sector. If the expected frequency for a uniform distribution (*e.g.*, 8.3% in each 30° sector of a circle) fell within these limits, the observed frequency is considered not significantly different (at the 1% level) from a random, non-oriented response. Otherwise, it is indicated in the figures as being above (plus sign) or below (minus sign) the expected value.

Note that this treatment was used instead of the attractive circular normal method (Batschelet, 1965; Waterman and Forward, 1970) because our data are generally not the normal, unimodal, continuous distributions appropriate to the latter. Furthermore most of our comparisons are made with normalized relative frequencies, not with the actual counts.

RESULTS

For all 24 fish on the two days 933 heading counts are available including all seven conditions; the corresponding number for the pauses, as defined above, is 636. For the 18 selected fish which were orienting while the sun's bearing was steady the total data are 775, the pauses 444. Consider first the circular distribution of the 18 fish on two days summed for all conditions (WP and NF) and plotted relative to North (Fig. 4A). Here all three sectors in the southerly quadrant are significantly preferred as is the sector centered at 30°.

Examination of the detailed data shows that the last sector's significance depends mainly on several runs on the afternoon of August 28. The pause criterion eliminates these counts, however, while reinforcing the southward preference (Fig. 4B). Indeed the pause count in the 30° sector in Figure 4B is significantly less than expected as are those in the other sectors from North to West. We can conclude then that the peak near NNE is behaviorally distinct as it is associated with more rapid turning by the halfbeaks. The broad southerly preference is characteristic of both the total counts and the pauses.

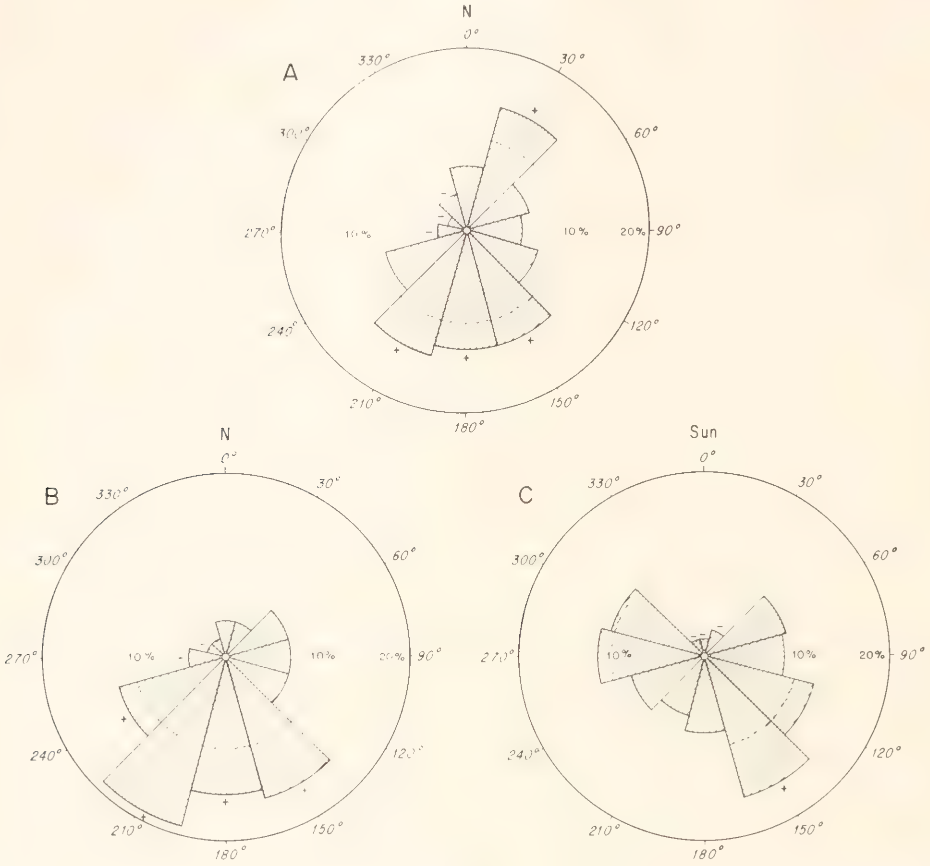

FIGURE 4. *Zenarchopterus'* directional responses relative to North and to the sun. A and B permit comparison of total (n = 775) and pause (n = 444) data (see text for definitions) plotted relative to North at 0°. B and C (n = 444) compare the pause data distributions relative respectively to North and to the sun's bearing. Eighteen fish, both days, seven conditions (one without polarizer and six different *e*-vector directions). Plotted as percentages of the numbers observed in twelve 30° sectors around 360°. Directions whose sectors contain greater percentages than expected at the 99% confidence level are indicated by "+" and those having less than expected by "–." The distribution in Figure 4C is dissected by days in Figure 5.

To analyze this geographic directional choice further the data need to be considered relative to the sun's bearing. When so plotted the pause distribution obtained for the 18 selected fish shows only one significant preferred sector at 150° clockwise from the sun's bearing (Fig. 4C). However, note that the whole quadrant centered in the solar direction is significantly avoided.

Since the sun's bearing differed by nearly 180° on the two experimental days (Fig. 2) this overall distribution needs to be dissected accordingly. The corresponding orientation patterns are quite different (Fig. 5A, B). Both are asymmetrical and skewed away from the sun. But in the morning data (Fig. 5B) the significant sectors are at 60°, 120° and 150° re the sun's bearing. Geographically these range from about SSE through SW. In contrast the afternoon data (Fig. 5A) show peaks at 240°, 270° and 300°. But their geographical distributions are about SSW, S and SSE. On both days the quadrant centered on the sun's bearing was significantly avoided as was one other quadrant which reference to the geographic coordinates identifies as northerly for each case. The apparent avoidance of the sun's general direction and the broad southward tendency which is shown by a preponderance of headings 90° to the sun's vertical indicate that the solar azimuth is involved in the observed responses.

These implications are strengthened by the oriented reactions to the NF condition where the fish were responding without a filter-imposed *e*-vector (Fig. 6A). Some counts appear in all but one sector (300° re N) but the only direction significantly preferred was 180° (South). In addition the southerly quadrant was preferred in 45% of the pause headings. Examination of the corresponding total count distribution for the 18 fish shows a similar pattern except that an additional significant peak is present at 30°.

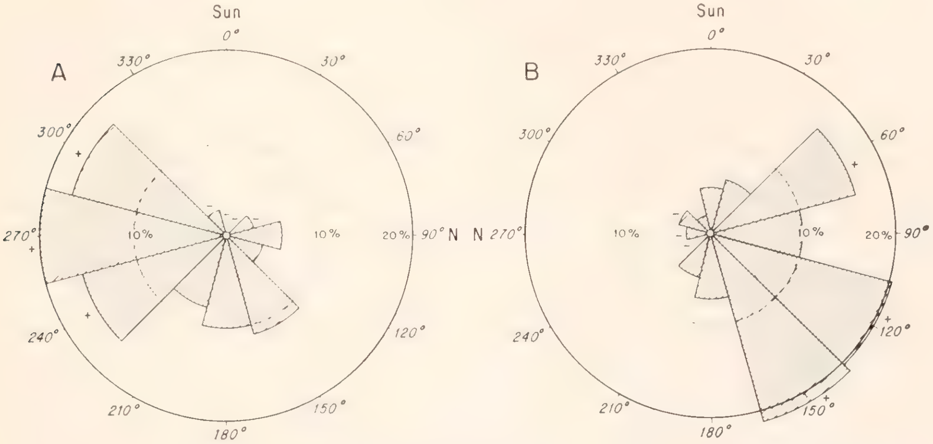

FIGURE 5. Comparison of *Zenarchopterus'* orientation relative to the sun on the two experimental days; (A.) August 28, 10 fish, n = 253; (B.) August 29, 8 fish, n = 191; pauses only, seven conditions (one without polarizer, six with different *e*-vector directions). Sectors and significant directions as in Figure 4. Figure 4C is the sum of the data in Figures 5A and 5B.

We have already seen that this is associated with faster turning and is eliminated by the pause criterion. The 24 fish total count distribution like the 18 fish pause count for NF has only one significant sector and that, too, is at 180°; the last four fish on the 29th reacting while the sun's bearing was changing rapidly showed no significant peaks in their NF distribution. Thus a strong southward orientation was evident in our *Zenarchopterus* data not only with the six WP conditions (randomized *e*-vector) plus NF but in the last condition alone where just the natural illumination was available.

Of course the blue sky visible in the NF condition has its own characteristic polarization pattern. A comparison between the NF response and that WP condition most closely simulating the natural pattern should therefore be instructive. The sky polarization in the zenith and all along the vertical great circle passing through the sun, *i.e.*, the sun's vertical, is perpendicular to the solar bearing. Consequently the 90° WP condition of the six used would no doubt most closely resemble the natural sky pattern.

In the latter, however, the degree of polarization, maximum about 90° from the sun, varies for blue sky from 50–90% depending on the earth's albedo, the turbidity of the atmosphere and the sun's zenith distance (Sekera, 1957). Near the surface, if the water is relatively calm as in our experimental vessel, this polarization as

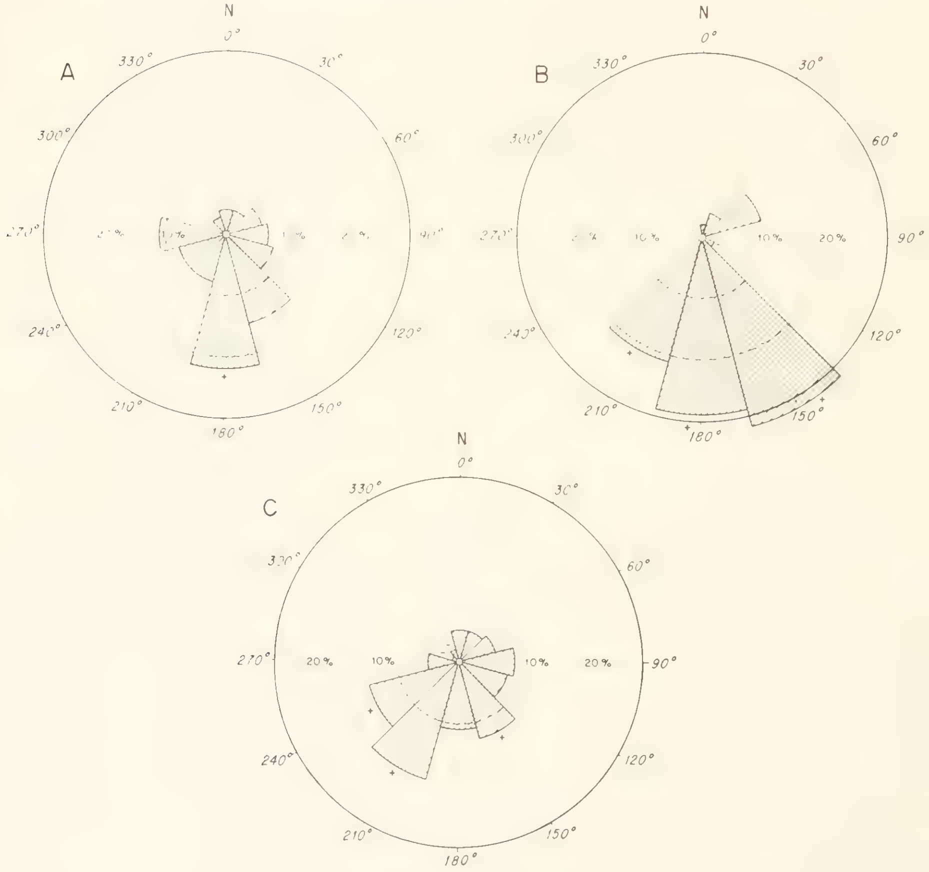

Figure 6. Comparison of *Zenarchopterus'* orientation relative to North under natural illumination and with different *e*-vector directions imposed by a polarizer; (A.) natural illumination without polarizer (NF condition), n = 96; (B.) with polarization plane at 90° to the sun's bearing (parallel to natural polarization in the sun's vertical), n = 53; (C.) with-polarizer runs for five of the six specific *e*-vector directions (WP at 0°, 30°, 60°, 120°, 150°). Note that WP 90° (Fig. 6B) is excluded here. See text for discussion, n = 295; Eighteen [illegible] only. Sectors and significant directons as in Figure 4.

well as that of the whole sky would be directly observed underwater through the critical angle (Waterman, 1954). In contrast our experimental 90° WP condition provided nearly 100% polarization of the visible light with the *e*-vector perpendicular to the sun's bearing apparent through the whole sky not just along the sun's vertical.

The corresponding halfbeak orientation with 90° WP indicates that more than half of the sectors were avoided and the three southward ones (approximately SSE, S and SSW) were significantly preferred at better than the 1% level (Fig. 6B). Indeed 82% of the headings in this distribution fall within the southerly quadrant.

Note that the approximately 65% reduction in luminous flux due to placing the polarizer over the experimental vessel did not alter the fishes' general behavior or azimuth preference when the imposed *e*-vector was parallel to that of the sky in the sun's vertical. Actually the resulting increases in the degree of polarization and the extension over the whole celestial hemisphere of the N-S *e*-vector direction naturally present in the sun's vertical are correlated with a reinforcement of the southward preference.

Because rather similar strong southerly preferences were demonstrated by the NF and 90° WP data one could argue that these two conditions (out of the seven tested) were sufficient to dominate the overall data distribution relative to geographical North (Fig. 4A, B). This is not the case, however. If all the pause data minus the NF and 90° runs are plotted, 44% of the headings are still in the quadrant centered on S (Fig. 6C). This is a significant preference over random for the four quadrants at better than the 1% level. The same was true of course for the NF and 90° WP plots themselves (Fig. 6A, B) but this tendency persists in the rest of the data, too (Fig. 6C). Therefore we can conclude unambiguously that the southward preference indicated in the total WP plus NF pauses is not due to the NF and 90° runs alone.

To test for any polarotactic effect of the imposed *e*-vector all responses to the six geographical directions of polarization plane produced by the Polaroid filter should be appropriately grouped and totaled. To permit this data were "zero corrected" by transposing counts so that the *e*-vector directions for all six WP conditions fall on the 0–180° plotting axis. Since polarized light is symmetrical about the *e*-vector axis, diametric responses, *e.g.,* 90° and 270° are equivalent (Fig. 2). Thus, if it perceives the polarization plane an organism can tell whether its anteroposterior axis is heading to the right or left of this *e*-vector. However, on the basis of that clue alone orientation would be subject to an 180° ambiguity around the horizon.

Heading responses were therefore folded around the axis perpendicular to the plane of polarization by adding diagonal pause counts. This yields a semicircular distribution counterclockwise (left) and clockwise (right) from the *e*-vector. Corrected in this way, a fairly strong and significant (at the 1% level) preference is shown for the 0° direction, *i.e.,* parallel to the *e*-vector (Fig. 7A).

The validity of this conclusion can be reinforced by showing that the selection of data for pauses sharpens the decided preference of *Zenarchopterus* for orientation parallel to the *e*-vector. Yet it does not alter the inferences which may be just as clearly drawn from the unselected total data for the 18 fish (Fig.

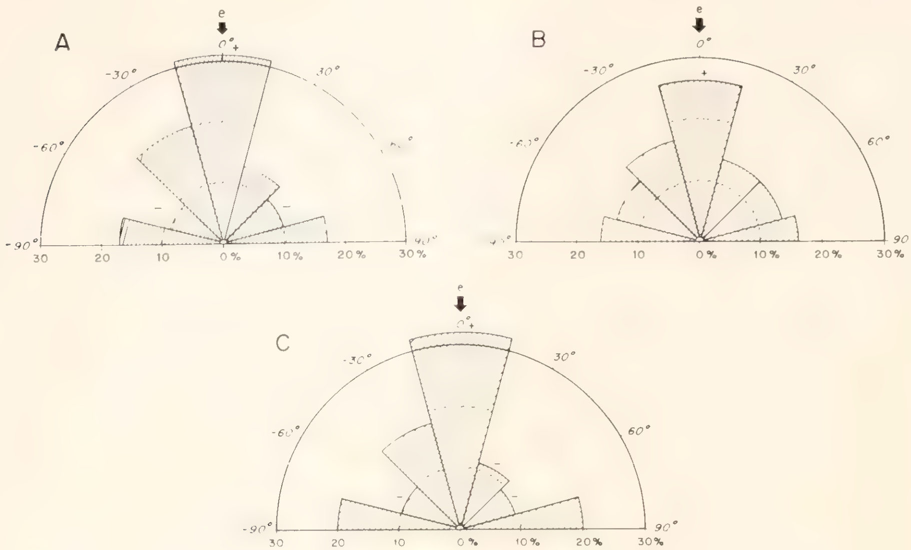

FIGURE 7. Polarotactic responses of *Zenarchopterus* to imposed linear polarization. (A.) data for all six tested *e*-vector directions (6 WP conditions) superimposed, pauses only, n = 348; (B.) same as Figure 7A but total counts used instead of just pauses, n = 578; (C.) same as Figure 7A but counts for WP 90° excluded; WP 0°, 30°, 60°, 120° and 150° included, n = 295; Eighteen fish. Sectors and significant directions similar to those of Figure 4 but data folded to 180° as described in text.

7B). There the distribution is somewhat more random, no sectors are significantly avoided and only 26% instead of 31% of the headings are parallel to the plane of polarization. Nevertheless the 0° sector is again the only one significantly preferred.

Finally we can show that the strong southerly preference of the 90° WP data (Fig. 6B) adds to but is not by any means the only support in the total WP data for orientation parallel to the *e*-vector. Thus subtracting the 90° WP headings from the overall WP distribution yields a new plot (Fig. 7C). However, its implications are exactly the same as for the selected pause data (Fig. 7A) and the total unselected headings (Fig. 7B). Again the only significantly preferred sector is that centered at 0° (Fig. 7C). This may be taken as evidence that *e*-vector direction can at least partially override orientation clues.

DISCUSSION

These results for *Zenarchopterus* demonstrate a time compensated menotaxis, a polarotaxis and, possibly, a negative phototaxis. The last is suggested by the significant avoidance of the sun's quadrant evident in distributions plotted relative to the solar bearing (Fig. 4C, Fig. 5A, B). However, in the absence of controls, other explanations of this are possible such as strong positive preference for different directions which would leave the solar quadrant relatively empty.

On the other hand the case for a time compensated menotaxis is well supported by the marked preference for southerly headings shown by the fish

(Figs. 4B, 6A, B, C). This must be time compensated because South was 90° counterclockwise from the sun on August 28 and 90° clockwise on August 29.

Preliminary observations indicated that the fishes' orientation was weak or absent when clouds obscured the sun even though only 35–40% of the sky was covered. This reinforces the conclusion supported by the heading distributions plotted relative to the sun's bearing (Figs. 4C, 5A, B) that the observed response is a *photo*menotaxis and not behavior mediated by another untested sensory modality *e.g.,* geomagnetic or geoelectric fields (McCleave, Rommel and Cathcart, 1971). In fact the relations between the response distributions obtained with the various conditions tested indicate that the preferences observed must be primarily visually determined. For example the four fish tested at the end of the August 29 sequence when the sun's bearing was changing rapidly were disoriented relative to the first eight fish run with a steady solar azimuth.

The menotactic directional preference was more precise for the 18 selected fish under natural illumination (NF) than in the totals for all conditions; for the NF distribution the only significant preference was due South at 180° (Fig. 6A). Similarly a preponderant number of headings occurred in the southerly quadrant for the 90° WP condition (Fig. 6B). Note, however, that even if these two conditions are subtracted from the total counts, the remaining data (WP at 0°, 30°, 60°, 120°, 150°) also show highly significant southward preference (Fig. 6C).

This preferred geographical direction perhaps relates either to the axis of the channel (150°–300°) at the collecting site (Fig. 1) or the direction (150° relative to North) which leads from the experimental area (E, Fig. 1) to the site of collection (H. Fig. 1). Previous underwater tests had also demonstrated menotactic preference for the directions approximately parallel to the channel of the fish's normal habitat (Waterman and Forward, 1972). More experiments are necessary to determine the validity of these several correlations.

It is not clear from the present results whether the fish are using the sun's position directly or the related sky polarization patterns to select their preferred geographical headings. The fact that *Zenarchopterus* tends to orient parallel to imposed *e*-vectors different from that in the sun's vertical (Fig. 7C) shows that they can and do orient to the plane of a superimposed polarization pattern. Also as noted above increasing the degree and area of overhead polarization having its *e*-vector parallel to that in the sun's vertical was correlated with a reinforced southerly orientation preference. This suggests but obviously does not prove that sky polarization plays a role in the responses of *Zenachopterus* to natural illumination.

However, the strong asymmetry of the present data's geographical distributions shows that more than the plane of imposed polarization must be involved. The *e*-vector is of course symmetrical through 180° and therefore by itself indicates pairs of opposite geographical directions. Perhaps there is an interaction between a polarotaxis symmetrical through 180° and a response dependent on the sun's asymmetric position.

As mentioned above the fish in preliminary experiments showed little or no orientation when the sun was obscured by clouds. A similar correlation also appeared in our underwater experiments with *Zenarchopterus* (Waterman and Forward, 1972). This finding may seem rather surprising in terms of the expectation that polarotaxis functions merely as a supplementary "sun compass" when the sun itself is obscured. However, our results may indicate instead or in addition that there is a releasing effect of the sun's disc (or the correlated high light intensity) on the underlying orientation preferences. Clearly such hypotheses must be tested by further experiments.

The strong polarotactic preference for orientation parallel to the *e*-vector of the downward illumination is unequivocal in these experiments. Essentially the same heading pattern appears in the data corrected to superimpose the *e*-vector whether the six pause distributions for different directions of the polarization plane are plotted (Fig. 7A) or the total WP data (Fig. 7B), or the pauses minus the 90° counts (Fig. 7C). Hence the observed behavior must be a polarotaxis and not just some other overriding response yielding southerly orientation.

This polarotactic orientation of *Zenarchopterus* swimming at the water surface differs in several ways from the distributions previously observed underwater (Waterman and Forward, 1972). Thus the present response to the *e*-vector appears considerably stronger in the total data than in the six day sequence of underwater experiments. The polarotactically preferred sector in the water surface experiments comprised 26% of the distribution (Fig. 7B) the corresponding sector in the underwater case contained only 19%.

However, in the underwater data selection of fish and using criteria for pauses increased the relative counts in the most preferred direction to 27% whereas the corresponding sector for the water surface experiments increased relatively less but reached 31% for the pauses (Fig. 7A). Consequently future experiments should most likely make use of this stronger orientation and the obvious practical advantages of the water surface type of tests. In addition the experimental situation at the water-air interface more closely mimics the environmental conditions usually experienced by this particular fish. Hence results like the present ones may be a better indicator of normal behavior.

A second difference between these and the previous experiments is that both perpendicular and parallel polarotaxis were observed underwater with the former being the more pronounced (Waterman and Forward, 1972). Only parallel polarotaxis is significantly preferred in the present results. Third, those submarine studies showed that strongest polarotaxis occurred to imposed *e*-vectors differing maximally in direction from that of the natural illumination in the sun's vertical (Waterman and Forward, 1972). This is not evident in the present data.

A fourth difference is that no oblique orientation to the *e*-vector is observed in the water surface results. In the underwater experiments the fish oriented either in the 0°, 90°, 180°, 270° quartet of directions or in one of the four oblique alternatives. Such bimodal preference patterns (parallel or perpendicular *vs.* oblique) are well known in both crustaceans and insects; corresponding four-peak preferences occur in cephalopods, too (Waterman, 1966). However, the responses of these other animals are most likely basitaxes evoked under particular experimental conditions rather than the freer menotaxes observed in the present experiments.

Our results reported above demonstrate both a menotactic azimuth preference which is parallel to the direction of naturally occurring polarized light in the zenith and the sun's vertical as well as a strong polarotaxis parallel to an imposed *e*-vector. The correlation observed between these two types of orientation is suggestive evidence that responses to polarized light may be involved in sun compass orientation by this fish.

The authors are grateful to Mabelita M. Campbell and William F. Corell for helpful assistance in carrying out and analyzing these experiments. In addition we would like to thank Mr. Peter Wilson and especially Mr. Robert Owen of Palau for their generous aid in making the field work practical.

SUMMARY

1. Visual orientation of the surface-living hemirhamphid teleost *Zenarchopterus* has been studied with individual fish swimming in an experimental vessel open to the air. Measurements of spontaneous heading preferences were made in the afternoon and morning respectively of two successive days, during which the sun's bearing differed by nearly 180°. Fish were tested under natural illumination of sun and sky as well as with six different *e*-vector directions of imposed linearly polarized light.

2. Data were selected among other things on the criterion that maintenance of a given azimuth direction ± 20° for a 10 sec period counted as an oriented response. Comparison with the distributions of the total measurements justifies this selection.

3. *Zenarchopterus* avoided the azimuth quadrant towards the sun. This suggests negative phototaxis but other explanations are possible.

4. A strong southerly heading preference occurred on both days under natural illumination by sun and sky. The same marked preference is also evident in the with-polarizer data plotted relative to North. This persists in the residual data when the counts are subtracted for the N-S imposed *e*-vector which parallels the sky polarization in the sun's vertical.

5. Such orientation occurred while the sun's bearings were constant; when solar bearings were changing rapidly orientation was less clear or absent. These results support a time compensated sun compass orientation.

6. Responses to imposed polarization patterns show a strong preferential orientation parallel to the *e*-vector. This persists when the N-S imposed *e*-vector counts (which demonstrate strong southerly preferences parallel to the sky polarization in the sun's vertical) are subtracted from the overall data. Comparison with previous underwater experiments on the same species indicates that these water surface data yield stronger polarotaxis and may provide better evidence for normal behavior.

7. The correlation of a menotactic azimuth preference parallel to sky polarization in the sun's vertical with strong polarotaxis parallel to the *e*-vector provided by a polarizer suggests that responses to natural polarized light may be involved in normal direction finding by *Zenarchopterus*.

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HORMONAL FACTORS IN THE CNS AND HEMOLYMPH OF PUPARIATING FLY LARVAE WHICH ACCELERATE PUPARIUM FORMATION AND TANNING[1]

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We formerly reported the existence of a hormone, derived from neurosecretory brain cells of fly larvae, which accelerates puparium formation (pupariation) in whole larvae or ligated hind parts. Apparently the same effect appeared when hemolymph (blood) from pupariating larvae was used in the place of brain extracts (Zdarek and Fraenkel, 1969). It was assumed at the time that the active factor in the blood was identical with, or directly derived from, the active component in the neurosecretion. Our assay system has been acceleration of tanning of the hind parts of larvae ligated after adequate ecdysone has been released to sustain pupariation. This critical period has been reached when the region around the posterior spiracles turns red. Since the central nervous system (CNS) in such ligated larvae is concentrated in the front part, the hind parts are totally paralyzed, and the only criterion for pupariation is tanning. From the few injection experiments with whole larvae it was learned that the accelerating effect applied not only to tanning, but the whole gamut of morphogenetic effects during puparium formation which precede tanning.

In a subsequent study of these morphogenetic events during pupariation (Zdarek and Fraenkel, 1972) the significance of certain early processes was recognized. These consist of a gradual slowing down of locomotion, an irreversible retraction of the anterior body segments into the body, and a gradual muscular contraction and cuticular longitudinal shrinkage into the barrel-shaped puparium.

The present investigation deals with the roles of substances in the brain and blood which promote anterior retraction at the beginning of puparium formation, and accelerate contraction and tanning. Surprisingly, the effects produced by injections of brain extracts and blood have turned out as not identical.

MATERIALS AND METHODS

All experiments to be described in the following were performed with the fleshfly, *Sarcophaga bullata* Parker, except where stated otherwise. Extracts from the CNS or hemolymph were injected into post-critical larvae in the red-spiracle stage, *i.e.*, 3–4 hours before the formation of the white puparium.

Basically, when testing the activity of various preparations we used 3 different criteria:

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[1] Supported by NSF grants GB 5441X and GB 23422, and NIH grant 5-K6-GM-18,495.

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(1.) *The retraction effect*

Blood- or brain fractions were injected into red-spiracle larvae, and the time was determined until the anterior body segments became retracted (Zdarek and Fraenkel, 1972). This time was then expressed as a percentage of the corresponding period in controls which had been injected with Ringer.

(2.) *Cessation of locomotion*

In the normal larva, retraction of the anterior end also signals the end of locomotion, since the mouthhooks play an essential part in crawling. However, under certain conditions, retraction is delayed, and is preceded by the contraction and shrinkage processes. The latter, then, make locomotion impossible.

(3.) *Acceleration of tanning*

Here, the time between injection into red-spiracle larvae of blood or brain extracts and the onset of tanning (darkening) was determined, and expressed as a percentage of the corresponding period in controls injected with Ringer.

In a variation of this test, the procedure, extensively used in our earlier work (Zdarek and Fraenkel, 1969), was followed. Injections were made into hind parts of red-spiracle larvae immediately after they had been ligated. As in our publication, the results were expressed as the quotient P/A between the time from injection to tanning in the posterior (P), and that in the uninjected anterior part (A).

The advantage of using intact larvae in the tanning test lies in the fact that anterior retraction and tanning can be determined in one and the same specimen. Complications arise when tanning is accelerated to the extent that it starts before, or simultaneously with anterior retraction. The advantage of using ligated hind parts is mainly the much greater ease and accuracy with which the beginning of tanning can be determined in the immobile ligated preparation, as against the mobile whole larva. The disadvantage is, of course, the impossibility of determining anterior retraction in the same preparation.

The brain-somatic ganglia complex which in fly larvae is concentrated in a single mass in the anterior part of the body was dissected from larvae or white or orange puparium stages, ground in water in a Potter-Elvehjem homogenizer, centrifuged, and injected into larvae or ligated hind parts of the red-spiracle stage, as described before (Zdarek and Fraenkel, 1969). Hemolymph was drawn from larvae or prepupae of different ages by puncturing with a very fine pipette, and was usually pooled from about 20 specimens before injection.

Brain extracts were injected in equivalents of between 1 and 4 brains per host larva, dissolved in 5 μg Ringer solution or distilled water. Hemolymph was injected in 4, 5, or 10 μl per host.

All figures in the tables or in Figure 1 represent mean values from 10 to 15 individual specimens.

EXPERIMENTS

(1.) *The effect of CNS-extracts*

Table I gives the results of an experiment in which the various processes which occur during puparium formation were observed in larvae injected with CNS-homogenates (2 CNS/larva) from red-spiracle larvae, or with hemolymph from approximately 1-hour old puparia (4 μl/larva), and compared with Ringer-injected controls. In this experiment each single individual was under observation from injection until onset of tanning.

The CNS-injected larvae started to contract after about 20 minutes and the contraction proceeded until the white puparium was completed about 40 minutes later. At first they were still crawling in a semi-contracted state, but they had virtually come to a stop by 37 minutes. The mouth hooks became withdrawn about 10 minutes later, shortly before the white puparium was completed. Tanning started soon afterwards. Each of these events took place in only a fraction of the time of that in the controls (10.5 to 32% for the different events), but the sequence was different under the two circumstances. In the controls, as in normal larvae, locomotion ceases with the withdrawal of the mouthhooks, and the contraction into the puparium follows later.

Table I

Sarcophaga bullata. *The effect of the injection into red-spiracle larvae of homogenates from the CNS of red-spiracle larvae, or of hemolymph from the 1-hour puparium stage, on the manifestation of various events during puparium formation. Time is in minutes after injection.*

	n	Become immobile*	Mouthhook withdrawn	Gradually contracting	White puparium	Tanning starts
Controls (Ringer injected)	9					
Average time		159	185	190	200	234
Range		120–240	125–260	130–265	135–270	165–360
2 CNS/larva	8					
Average time		37	47	20	60	75
Range		20–60	35–65	15–25	45–75	50–120
% of controls		23.3	25.4	10.5	30	32
Hemolymph (4 μl/larva)	9					
Average time		16.1	20.5	25	30	85
Range		15–20	20–25	20–30	25–40	65–130
% of controls		10.1	11.1	13.2	15	36.3

* Controls and hemolymph injected: Immobilization caused by anterior retraction. CNS: Immobilization caused by beginning contraction.

In another experiment with 2 CNS/larva, the effect of CNS-homogenates from larvae or prepupae of different ages was compared. On this occasion the criteria were termination of locomotion and onset of tanning in unligated specimens, and tanning in ligated specimens (Table II). It appears that the activities of extracts in relation to these criteria differed relatively little in the CNS of different developmental stages, from the mature larva to 24 hours after pupariation. Irrespective of whether the observed differences in the figures are significant, it is clear that the CNS retains a considerable activity in all the developmental stages investigated.

Essentially the same results ensued from a similar set of tests where only one CNS/host was used (Table II). Here the activities of the extracts apparently had decreased at 24 hours after pupariation in tests with unligated larvae, but no such decrease had occurred in tanning tests with ligated larvae. Extracts from only 1 CNS were as active as extracts from two.

When the equivalents of 4 CNS were injected into each larva, contraction into the puparium proceeded even faster and the larvae seemed to be overcome by tanning before they had managed to retract the anterior ends. The mouthhooks in such puparia either remained outside, or could be pushed outside by squeezing the newly formed puparium.

TABLE II

Sarcophaga bullata. *The effect of the injection of homogenates from the central nervous system, taken from larvae or prepupae of different ages, into red-spiracle larvae, on the onset of immobilization and tanning during puparium formation. These effects are expressed as a percentage of the time after injection in which these events occur in the ringer-injected controls*

2 CNS/host				1 CNS/host			
Donor	Immobilization % of control	Tanning		Donor	Immobilization % of control	Tanning	
		Unligated % Control	Ligated P/A§¶			Unligated % Control	Ligated P/A§+
Mature larva	46	34	0.47	Mature larva	39	31	0.44
Early red-spiracles*	39	28	0.46	Red-spiracles***	37	33	0.56
Late red-sp. 1.**	47	27	0.37				
0-hr†	38	27	0.51	2-hr.†	32	32	0.47
4-hr†	43	30	0.43				
8-hr†	45	37	0.40	14-hr.†	36	39	0.60
16-hr†	38	45	0.49				
24-hr†	62	50	0.50	24-hr.†	51	46	0.60

* 3–4 hours before white puparium.
** 1–2 hours before white puparium.
*** 2–3 hours before white puparium.
† After white puparium.
§ Period between injection and onset of tanning in posterior (P) and anterior (A) part.
¶ Control 1.67.
+ Control 1.52.

In another experiment, CNS's from younger fully grown larvae with the crop still full were tested, and there was no difference in activity between them and those from red-spiracles larvae.

(2.) *The hemolymph*

Injection of hemolymph from orange puparium stage (about 1 hour after the white puparium stage) led to an almost abrupt cessation of locomotion after 15 to 20 minutes. This period was in the experiment of Table I only 10.1% of the time in the Ringer injected controls. In other experiments, injection of hemolymph produced immobilization in 18, 19, 20, 27 and 28%, respectively, of the time in the controls. Such differences were most probably due to differences in the state of development of red-spiracles larvae, and in temperature.

This immobilization was caused by the irreversible retraction of the anterior segments, and was followed by the gradual contraction into the puparium, and subsequent tanning. All these processes were enormously speeded up, by comparison with the same processes in the controls. The sequence of events after injection of blood was the same as in normal larvae or Ringer-injected controls. This differs fundamentally from the sequence after injection of CNS, as described above and also presented in Table I, where immobilization signals the beginning of contraction, and where anterior retraction occurs either during or after completion of the contraction process.

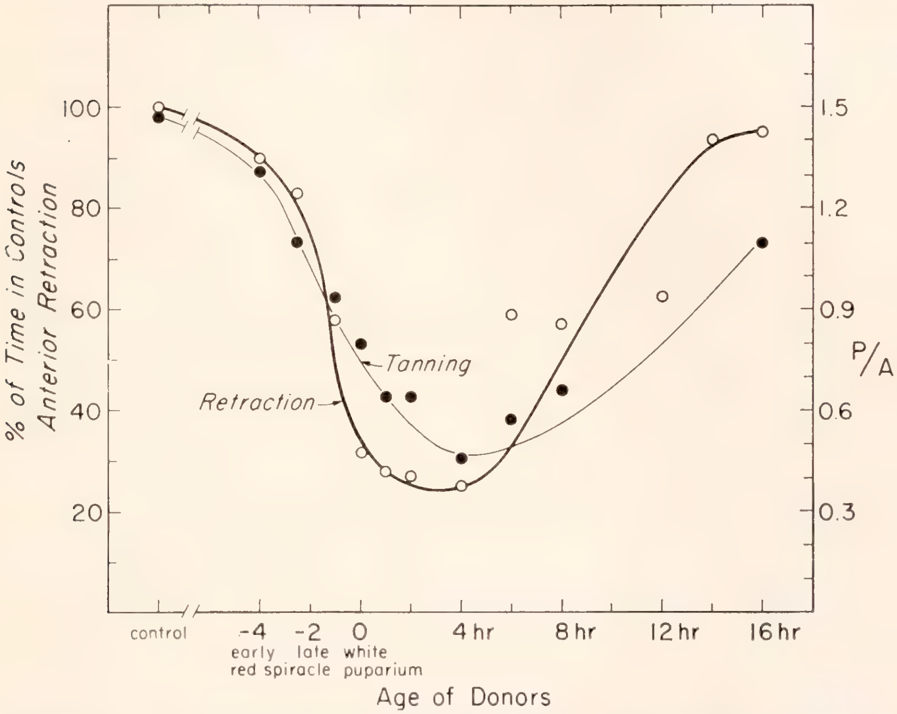

FIGURE 1. The accelerating effects on pupariation in larvae of *Sarcophaga bullata,* caused by the injection of hemolymph from larvae in different states during puparium formation. The heavy line represents the acceleration of retraction of the anterior end after injection into whole red-spiracles larvae. The effect is expressed as a % of time in the controls. The thin line represents the acceleration of tanning in ligated hind parts. The effect is expressed as the quotient of the period between injection and tanning in the injected posterior (P), and the (non-injected) anterior (A) parts. Each point represents an average from 10 larvae.

The white puparium stage is reached much sooner after injection of hemolymph, than after that of CNS-homogenates. Tanning, however, starts at about the same time in both cases, or, if anything, sooner in the latter case. The time interval between injection and onset of tanning was in both cases about one-third that in the controls (Table I).

Injection of active hemolymph into younger larvae which had already emptied their crop but showed no sign yet of red spiracles had little or no effect.

Figure 1 shows the results of the injection of hemolymph from donors of different ages. The hemolymph at the early red-spiracles stage, about 4 hours before pupariation had little or no effect on retraction or tanning. Towards the white puparium stage it reached a peak of activity which was maintained for about 4 hours. All tests with hemolymph from orange puparium stage fall under the category of high activity. The retraction effect then decreased sharply and had about disappeared 14 hours after pupariation. The tanning effect also declined but less decisively than the retraction effect, and was still considerable at the 16-hours point. This difference in the decline of these two effect shows, as we shall see later, that the two activities are not identical.

TABLE III

Sarcophaga bullata. *The effect of the injection into intact or ligated hind parts of red-spiracle larvae of different dilutions of hemolymph from the orange puparium stage on acceleration of anterior retraction before, and tanning after pupariation*

Dilutions of hemolymph (10 μl)	Acceleration of	
	Anterior retraction whole larvae % of controls	Tanning ligated larvae $\frac{P}{A}$
Undiluted	19	0.76
1:1	24	0.85
1:2	29	0.96
1:4	29	1.31
1:6	30	—
1:8	52	1.30
Ringer	100	1.34

(3.) Effects of different fractions of blood

The hemolymph from orange puparia was centrifuged to separate blood cells from plasma, and both fractions were injected separately. The blood cells were suspended in the original volume of Ringer. All the activity, both as regards acceleration of tanning and retraction, resided in the plasma fractions, while the blood cells were entirely inactive.

(4.) Effect of dilution of blood

Plasma from orange puparia was tested in a series of dilutions, up to 8 times. With a 4-times dilution the effect on tanning had entirely disappeared, while the retraction effect was still strong with even an 8-times dilution (Table III).

(5.) Specificity of the hemolymph effects

The hemolymph (plasma) from white puparia of 3 species of flies, *Sarcophaga bullata, S. argyrostoma,* and *Calliphora erythrocephala* was tested in *S. bullata* hosts and found equally active, both as regards the retraction and tanning effects.

(6.) *Origin of the active substances in the blood*

We have seen that the hemolymph of fly larvae in the red-spiracle stage a few hours before pupariation entirely lacks any activity in regard to the acceleration of retraction and tanning, and becomes active only at the time of pupariation. We had at first assumed that the principle which accelerates tanning was released into the hemolymph as a neurohormone, originating from the median neurosecretory cells in the brain (Zdarek and Fraenkel, 1969), and there was no *a priori* reason to doubt that the same applied to the retraction effect which was only recognized later. However, the following experiments show that both activities can arise in the hemolymph in the entire absence of the CNS. This was the result of tests with hemolymph from hind parts of larvae which had been ligated at a stage when it lacked such activities.

TABLE IV

Sarcophaga bullata *and* S. argyrostoma. *The effect of the injection of hemolymph from the orange puparium stage of intact larvae or orange colored hind parts, into intact, or ligated hind parts of red-spiracle larvae, on acceleration of anterior retraction before, and tanning after purariation*

Donors (10 µl hemolymph)	Acceleration of	
	Anterior retraction whole larvae % of control	Tanning ligated larvae $\frac{P}{A}$
S. bullata intact	24	0.74
Hind parts (ligated in red-spiracles stage)	24	0.76
S. argyrostoma intact	25	0.65
Hind parts (ligated precritically, pupariation induced by ecdysone)	19	0.89
Ringer control (*S. bullata*)	100	1.28
Ringer control (*S. argyrostoma*)	100	1.68

The experiments were performed with hind parts of *S. bullata* and *S. argyrostoma* which had been prepared in different ways. Those of *S. bullata* were ligated in the red-spiracle stage, and the blood was taken when the hind part had reached the orange stage. Those of *S. argyrostoma* were from specimens which were wet-treated for 5 days, then injected with ecdysone, ligated and transferred to the dry. (Wet-treatment inhibits the release of ecdysone, and thus prevents pupariation; Ohtaki, Milkman and Williams, 1968; Zdarek and Fraenkel, 1970). Blood for injection was taken from these treated hind parts when they had reached the orange stage. The hemolymph of these hind parts, which was inactive at the time of ligation, and had remained separated from the source of neurohormones in the front part, had become active by the time the cuticles had turned yellow (Table IV) exactly as in intact specimens (Fig. 1).

This shows that both these activities, acceleration of retraction and tanning, can arise in the blood in the absence of the CNS at the time when tanning starts.

We have already shown above (Fig. 1) that blood from normal white puparia already contains these two activities, and that, therefore, tanning is not a prerequisite for their manifestation. The following experiments again show that the absence of visible tanning in no way interferes with the appearance of the retraction factor in isolated abdomens. Injection of α-MDH into red-spiracles larvae does not interfere with the formation of the puparium, nor with the first processes of stabilization of the cuticle, but inhibits the subsequent processes of visible tanning (Zdarek and Fraenkel, 1972). [α-MDH [(DL)-α-Methyl-α-hydrazino-β-(3,4 dihydroxyphenyl) propionic acid (Merck, Sharp, and Dohme)] inhibits DOPA decarboxylation, and thus tanning in adult flies and puparia (Seligman, Friedman and Fraenkel, 1969)]. This reaction was used in testing for a possible relation between tanning and the appearance of the retraction and tanning effects. α-MDH was injected into red-spiracle larvae and their blood tested after the puparia had formed. Treatment with α-MDH which prevented tanning in no way interfered with the appearance of the retraction effect.

TABLE V

The effect of various treatments of hemolymph from the orange puparium stage of Sarcophaga bullata *on anterior retraction before pupariation*

Treatment	Anterior retraction % of control	Description of effect
Heating at 80°, 10 m (filtrate)	100	heat labile
Dialysis, 24 hours	31	non-dialyzable
Freezing (16 hours)	26	remains active
Freeze drying	28	remains active
ETOH precipitated, supernatant and precipitate tested separately	100	activity lost
Acetone precipitated, supernatant and precipitate tested separately	100	activity lost
50% $(NH_4)_2SO_4$ precipitated, filtrate	100	activity lost
precipitate	about 40	remains active
TCA precipitated (filtrate)	72	some activity remains

The same result ensued in an experiment where red-spiracle larvae were injected with α-MDH, ligated, and the blood from the hind parts tested at the time when they would have started to tan in the absence of α-MDH. Since no puparial contraction occurs in the ligated hind part, and since tanning is inhibited by α-MDH, no visible change occurred in these preparations. The retraction factor appeared in these hind parts at the same time, and to the same extent as in ligated pupariating hind parts which had started to tan.

(7.) *Characteristics of the accelerating factors from blood and CNS*

A first attempt was made to characterize the chemical nature of the retraction factor from hemolymph (Table V). Hemolymph collected from orange puparia was submitted to a number of treatments, and then tested. The activity was entirely destroyed by heating blood at 80° C for 10 minutes. It was stable to freezing and freeze-drying and proved non-dialyzable. It was precipitated by alcohol or acetone with a total loss of activity.

After precipitation with 20% trichloroacetic acid, some activity remained in the filtrate. Precipitation with half-saturated ammonium sulfate removed the whole activity into the precipitate, from which it could essentially be recovered, after redissolving in water and dialyzing.

Similar preliminary tests were performed on the nature of the tanning accelerator in homogenates of the CNS, using P/A test. The results are not strictly comparable to those obtained with hemolymph, considering the differences in preparations, test procedure and, presumably, relative concentration, but show very similar features. The activity from the CNS is relatively stable to heat and freeze drying, is non-dialyzable, and precipitates in 10% TCA and a half-saturated solution of ammonium sulfate. It is destroyed by treatment with pronase and trypsin, but stable to pepsin.

All these tests suggest that the active components in hemolymph and CNS have similar characteristics, and that we are dealing with proteinaceous substances which are easily destroyed by the usual denaturation (and hydrolyzation) treatments.

Discussion

In an earlier publication (Zdarek and Fraenkel, 1969) we had established a neurohormonal effect, deriving from the pars intercerebralis of the brain, which accelerates puparium formation in whole or ligated fly larvae when applied after the critical period of ecdysone release. A similar effect ensued from the injection of hemolymph (blood) from pupariating larvae. It was at first assumed that this neurohormone was released from the brain into the blood.

Subsequently a detailed study of the morphogenetic events which comprise the act of pupariation was made (Zdarek and Fraenkel, 1972). This revealed several distinct processes of which the following are the most relevant for the present discussion: (1.) The irreversible retraction of the first 3 anterior segments into the body, (2.) A longitudinal contraction of the body muscles, (3.) A longitudinal shrinkage in the cuticle resulting in the smooth surface of the puparium, (4.) Tanning. In the present study a comparison was made between the effects of injections of CNS-material and of the hemolymph from larvae or prepupae of different ages, on the different processes which occur during pupariation. Any interpretation of the many-fold processes which take place between the release of ecdysone and the finished puparium will have to reconcile and integrate the following observations:

(1.) The activity of the CNS changes little, if at all, from the red-spiracle larvae through the 16-hours puparium stage. The blood is inactive at the beginning of the red-spiracles stage and reaches a maximum activity towards the white puparium stage. This activity is maintained for several hours and then declines and disappears.

(2.) Similar accelerating effects ensue from the injection of CNS-extracts or hemolymph, but they are not identical. CNS-extracts exert their strongest effect on puparial contraction and tanning, while the hemolymph at the peak of its activity has the strongest effect on anterior retraction.

(3.) The active substances in the hemolymph which accelerate anterior retraction on the one hand, and contraction into the puparium and tanning on the other cannot be identical. The retraction activity is far more dilutable than the tanning activity. This is not due to a lower threshold for the former because the hemolymph 16 hours after pupariation still shows a considerable tanning, but no retraction activity.

(4.) The 4 distinct morphogenetic processes we have mentioned above are acted upon by at least two different entities, one applying to the anterior retraction (X_r), and the other to the remaining group of processes comprising the formation of the puparium and tanning (X_t). No evidence has so far come to light which would suggest X_t to involve more than one substance.

(5.) The different morphogenetic processes which occur during normal pupariation in a temporally ordered fashion (Zdarek and Fraenkel, 1972) are not chain reactions, but can occur and be influenced independently from each other. Both X_r and X_t can arise in the absence of tanning (after α-MDH treatment). Acceleration of tanning occurs in the ligated hind part in the absence of all the other events. Contraction into the puparium can precede anterior retraction (by injection of CNS-extracts).

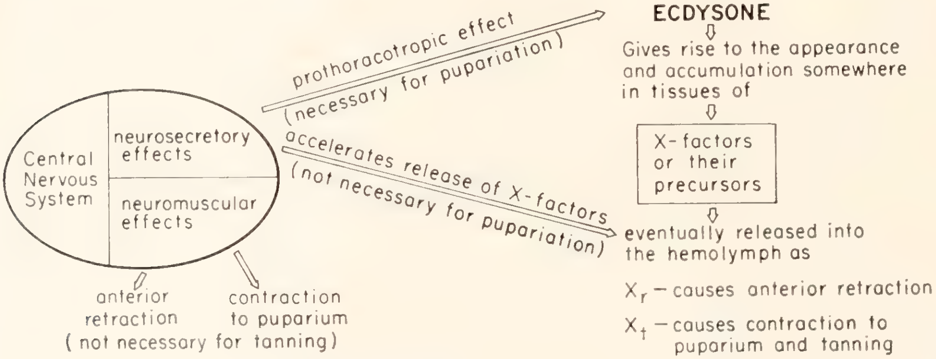
ECDYSONE
Gives rise to the appearance and accumulation somewhere in tissues of
X-factors or their precursors
eventually released into the hemolymph as
X_r – causes anterior retraction
X_t – causes contraction to puparium and tanning
prothoracotropic effect
(necessary for pupariation)
Central Nervous System
neurosecretory effects
neuromuscular effects
accelerates release of X-factors
(not necessary for pupariation)
anterior retraction
contraction to puparium
(not necessary for tanning)

FIGURE 2. Scheme of the interrelationships between neurosecretory and neuromuscular effects from the CNS, ecdysone, and the appearance of the X-factors in the hemolymph, during puparium formation of flies.

(6.) Both accelerating activities X_r and X_t can arise in the blood in the absence of the CNS (in the hind part ligated after the critical period, or ligated before, and injected with ecdysone).

(7.) The factors in the hemolymph and CNS which we have designated as X_r and X_t are most probably proteins and of large enough size to be nondialyzable.

The following is an attempt to draw up a hypothetical scheme which accounts for all these many-fold and sometimes seemingly contradictory and probably largely hormonal relationships (Fig. 2).

It starts with ecdysone the release of which is activated by a prothoracotropic neurohormone from the CNS. Ecdysone then causes the appearance of two proteinaceous substances which at first must be bound or contained in some tissue present in all parts of the body (possibly epidermis or fatbody), and ultimately are released into the hemolymph. One of them controls the retraction of the anterior segments (X_r), and the other affects the other morphogenetic processes, muscular contraction, cuticular shrinkage, tanning, which comprise puparium formation (X_t). The release of these two factors into the blood is stimulated or activated or accelerated by a neurohormone from the pars intercerebralis of the brain but occurs spontaneously and at a slower rate in the absence of the CNS.

This scheme seems to account for all observations described in this paper. In the normal red-spiracle larva ecdysone is present, and has already given rise to X_r and X_t in a location other than the hemolymph. Injection of CNS-homogenate accelerates the release of X_r and X_t into the blood, and thus accelerates the different processes which lead to pupariation. A ligature in the red-spiracle larva leads to earlier pupariation in the anterior part than the posterior, because the former remains subjected to natural activation by the CNS; injection of CNS material into the posterior part causes it to tan first (Zdarek and Fraenkel, 1969), because of the plenteous addition of the neurohormone. Injection of active hemolymph into normal red-spiracle larvae, or ligated hind parts of such larvae, has the same accelerating effect because the injection provides already liberated X_r and X_t.

The hind part of post-critically ligated larvae contains ecdysone, and X_r and X_t are already present but not yet released into the blood. The blood is at first inactive, but eventually becomes active. The hind part of a pre-critically ligated larva is either lacking in ecdysone, or may contain it in subeffective doses (Fraenkel and Zdarek, 1970; Zdarek and Fraenkel, 1970). Injection of ecdysone leads to tanning because it first gives rise to X_r and X_t in some tissue (as in the red-spiracles larva), with subsequent release into the blood. Thus the blood in the hind part becomes active although it had been separated from the CNS at a time when it was still inactive.

This scheme postulates the existence in active blood of two different proteinaceous factors, and a fundamental difference in the nature of the accelerating agent(s) in the CNS and hemolymph. It is hoped that further work into the isolation of these factors will bear out these conclusions.

A possible alternative to the above scheme is based on the assumption of the essential identity of the accelerating factors in the CNS and hemolymph. This would imply that a product of neurosecretion is stored in the peripheral nerves or at the nerve endings during and even before the critical period, and ultimately released as the X-factors into the hemolymph through the action of ecdysone. Injection of brain extracts would accelerate pupariation by putting the X factors into the hemolymph sooner than they would appear otherwise. Evidence for the working of such a scheme would be demonstration of the accumulation in or disappearance of neurosecretory material from nerves or nerve endings at appropriate times, and that of an identity of purified active substances which have been isolated from the CNS and hemolymph. The possible existence of such a neurohumoral transfer system in the neuromuscular synapses of insects, including a fly larva, can be deduced from the work of Osborne (1964, 1967) and Osborne, Finlayson and Rice (1971). Whitten (1963) actually interpreted histological changes in the dorsal median nerve of fly larvae at the time of pupariation as the movement of neurosecretory granules. These nerves disappear soon afterwards, after having fulfilled their presumed function in puparium formation. It is not obvious how these findings by Whitten can be applied to the case of the pre-critically ligated hind part where injection of ecdysone leads to the appearance of the X-factors in the hemolymph.

Either scheme postulates the existence of two proteinaceous substances interposed between ecdysone and its visible effects in puparium formation. If our reasoning underlying the scheme drawn in Figure 2 is correct, it should be possible to induce pupariation by injecting purified X-factors into a hind part which was ligated before the critical period, in the absence of ecdysone. If the alternative explanation holds, injection of active material from the CNS into a like preparation could achieve the same effect. Work on the isolation of active material from hemolymph and CNS is now in progress and will, hopefully, make the execution of these experiments possible.

These conclusions shed new light on a postulate made by Ohtaki, Milkman and Williams (1968) and subsequently elaborated and verified by us (Zdarek and Fraenkel, 1970), according to which the action of ecdysone in the formation of the fly puparium implied the gradual accumulation of "covert" effects within a target organ, produced by a cumulative effect of subeffective doses. It appears that our present results on the formation, accumulation, and release into the hemolymph of the X-factors exactly conform to these former observations, and indeed give a most satisfactory explanation for them. By this reasoning, the covert effects in the former studies might be nothing but the processes, set in motion by ecdysone, leading to the appearance in the hemolymph of the X-factors and finally to pupariation.

Puparium formation is, of course, a very special case among insects, but it would be surprising if the classical function of ecdysone in molting and metamorphosis would not ultimately also turn out to work through further groups of proteinaceous substances.

After this paper was first submitted for publication a paper by Kambysellis and Williams (1971) appeared in the pages of this journal with information highly relevant to our own conclusions. Both ecdysone and a "macromolecular" factor are required for successful spermatogenesis in a silkworm in vivo or vitro, but unlike our case where the proteinaceous X-factors appear in the blood in consequence of an action of ecdysone and conceivably exert their action in its absence, in their case the macromolecular factor is already present in the blood and carried to the site of action by ecdysone. In both cases the proteinaceous factors exert the ultimate effect.

SUMMARY

1. Hemolymph or central nervous system (CNS) homogenates from *Sarcophaga bullata* larvae in various stages during puparium formation were injected into red-spiracle larvae (due to pupariate within a few hours), where they cause an acceleration of pupariation. The predominant effect of CNS is on puparial contraction and tanning, and that of hemolymph on retraction of the anterior end.

2. The activity of CNS-preparations changes little from the mature larva through the 24-hours puparium stage. The activity in the hemolymph is absent up to 4 hours before pupariation, at a peak from the white puparium through 4 hours later and then declines. Sixteen hours after pupariation the effect on retraction has disappeared, while that on contraction and tanning is still considerable

3. The active substances in CNS and hemolymph which accelerate retraction or contraction/tanning are not identical and have been designated as X_r and X_t, respectively.

4. Both X_r and X_t can appear in the blood in the absence of the CNS, *viz.* in the hind part ligated after the critical period, or ligated before that period and injected with ecdysone.

5. The X-factors in CNS and hemolymph are of the nature of proteins. They are denatured by heat, alcohol, or acetone, precipitable by TCA and $(NH_4)_2SO_4$, non-dialyzable, and destroyed by trypsin or pronase (tested only for CNS).

6. These observations fit a scheme whereby ecdysone causes the appearance and/or accumulation of the X-factors in some tissue and their ultimate release into the hemolymph where they induce pupariation. A product of neurosecretion in the CNS accelerates the release. An alternative explanation assumes that the X-factors originate as a neurosecretion which is stored in the peripheral axons or synapses prior to their release into the hemolymph by the action of ecdysone.

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STUDIES ON THE NATURALLY OCCURRING HEMAGGLUTININ IN THE COELOMIC FLUID OF AN ASCIDIAN

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Recent studies on the immunological response revealed that an early indication of the appearance of adaptive immunity was found in one of the oldest types of vertebrates, a cyclostome (Good and Papermaster, 1964). In invertebrates, although their exact mechanisms remain obscure, there are many immunelike phenomena, for example, hemolysis, hemagglutination, bacteriolysis and bacterioagglutination by coelomic fluid, and the recognition of "self" or "not-self" by phagocytic cells (Huff, 1940, Briggs, 1966). The question whether the immune systems of vertebrates developed from one of the immunelike phenomena of invertebrates has not yet been solved (Burnet, 1968).

Although natural hemagglutinin can be found in the coelomic fluid of many invertebrates, its chemical and biological properties have been poorly understood (Marchalonis and Edelman, 1968; Makay, Jenkin and Rowley, 1969; Acton, Bennett, Evans and Schrohenloher, 1969). The discrimination between "self" and "not-self" by phagocytes was also observed in many invertebrates (Cameron, 1932; Aub, Tieslau and Lankaster, 1963), but its mechanism remains to be analyzed.

Data presented in this paper indicate the presence of hemagglutinin in the coelomic fluid of ascidians, uniquely placed between invertebrates and vertebrates. We then discuss its properties in relation to those of vertebrates.

MATERIALS AND METHODS

Two species of ascidians, *Styela plicata* (Lesueur) and *Halocynthia hilgendorfi* f. *ritteri* (Oka) were used. They were harvested at Noto Marine Laboratory in Ishikawa, Japan. The coelomic fluid was collected by cutting the test and mantle without injuring the internal organs. After removing the cells by centrifugation, the coelomic fluid was collected and stored at −15° C.

The coelomic fluid was dialyzed to saline of an appropriate concentration used for the erythrocyte preparation from each animal. The red blood cells (RBC) from various animals were centrifuged at 450 × *g* and suspended in veronal buffered saline, pH 7.2. The final concentration of RBC was adjusted to 10% (v/v, packed cells). The number of RBC of 10% (v/v) per ml from various animals were as follows; mice ($C_3H(He)$), 1.04×10^9; guinea pig, 1.02×10^9; sheep, 4.07×10^9; rabbit, 8.3×10^9; rat, 1.5×10^9; *Crassius carassius*, 4.23×10^8; *Bufo vulgaris*, 1.03×10^8; *Matrix tigris*, 3.15×10^8. To 0.3 ml of the 2-fold serially diluted coelomic fluid, 0.05 ml of red blood cells was added. After mixing completely, the hemagglutinating plate (Tomiki) was incubated at 37° C for 1 hour and the hemagglutination was observed.

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For absorption, packed erythrocyte washed several times with saline was added to an equal volume of coelomic fluid which had been previously dialyzed to the same saline. After incubation at 37° C for 1 hour with occasional shaking, the mixture was centrifuged and the supernatant was used for hemagglutinin test. The secondary and tertiary absorptions were also tested.

Sephadex G-100 or G-200 (Pharmacia) was equilibrated by 0.02 M phosphate buffered saline (pH 7.8). Before filtration, the coelomic fluid was heated at 100° C for 20 minutes, centrifuged and then dialyzed to phosphate buffered saline over night. For trypsin digestion, the reaction mixture used was as follows: 0.1 ml of 1–5 mg/ml trypsin (Difco, 1:250), 0.3 ml of dialyzed coelomic fluid, 0.1 ml of 0.2 M phosphate buffer pH 7.8. The reaction mixture was incubated for 30 minutes, 2 hours and 4 hours at 37° C.

Periodate treatment was done as follows: the dialyzed coelomic fluid was incubated at 25° C for 3 hours with the various concentrations of periodate which was adjusted to pH 5.4 with 2 N NaOH. Then the coelomic fluid was again dialyzed to saline and its hemagglutinic activity was estimated.

For the observation of phagocytosis, the coelomic fluid was poured out onto a cover glass by cutting the test and the mantle. After 10 minutes, the supernatant was discarded and coleomic cells adhering to the glass were used for observation. After washing with sea water several times the cells were fixed with glutaraldehyde at the final concentration of 1%. After the fixation had been carried out for about 1 hour, the preparation was washed several times by distilled water and then stained with Giemsa solution (× 10) for 20 minutes.

For other observations on phagocytosis, the coelomic fluid was poured out onto the plastic dish (Falcon) and after 10 minutes, the rabbit erythrocytes (fixed with 1% glutaraldehyde) were added. Twenty minutes later, the cells were fixed with 1% glutaraldehyde.

Results

The occurrence of hemagglutinin

Specificity of hemagglutinin. Rabbit erythrocytes were shown to aggregate when they were mixed with ascidians' coelomic fluid, even at a high dilution. The titer of hemagglutinin of *S. plicata* to rabbit erythrocyte was $+ 2^{13}$ and that of *H. hilgendorfi* was $+ 2^{12}$. The erythrocytes of fish, frog, and snake were not aggregated by the coelomic fluid of either *S. plicata* or *H. hilgendorfi*. The hemagglutination seems to be restricted to erythrocytes of mammals, such as mice, rats and rabbits. The erythrocytes of sheep and guinea pig, although these are mammals, did not aggregate in the presence of ascidians' coelomic fluid. There are some variations in the agglutinating activity among species of ascidians. The coelomic fluid of *H. hilgendorfi* aggregated rat erythrocytes (titer: $+ 2^{7}$) but did not aggregate that of *S. plicata*. Conversely, the coelomic fluid of *S. plicata* aggregated mouse erythrocytes (titer: $+ 2^{4}$) but did not aggregate that of *H. hilgendorfi*.

The absorption test was carried out to determine whether the hemagglutinin adhered to rabbit erythrocytes and disappeared from the supernatant as with mammalian antibodies or not. Two milliliters of the coelomic fluid of *S. plicata* were mixed with an equal volume of packed rabbit erythrocytes (1.8×10^{10} cells). After incubation at 37° C for 1 hour, the mixture was centrifuged and the supernatant was used for the hemagglutinin test (first step). The secondary and tertiary absorptions were done by the same procedure. After the tertiary absorption, the supernatant no longer aggregated rabbit erythrocytes. As described later, the coelomic hemagglutinin did not change its activity during an hour of incubation at 37° C.

The specificity of hemagglutinin was studied by the absorption method (see Table I). Tests were performed to determine whether the hemagglutinin for rabbit erythrocyte was identical to those of other animals and whether the hemagglutinin for rat or mouse erythrocyte was identical to that for rabbit erythrocyte. Two ml of the coelomic fluid of *S. plicata* and *H. hilgendorfi* was mixed with an equal volume of packed erythrocytes. The number of packed cells of several animals per ml were as follows; rat, 1.3×10^{10}, mice (C_3H), 1.1×10^{10}, rabbit, 8.3×10^{9}. After incubation at 37° C for 1 hour, the mixture was centrifuged and the supernatant was used for hemagglutinin test (primary dilution, × 2). The secondary (dilution, × 4) absorption was done by the same procedure. The questions of whether the hemagglutinin of *H. hilgendorfi* is equally effective for the aggregation of rat and rabbit erythrocytes, and of whether the agglutinin against mouse erythrocytes is identical to the hemagglutinin to rabbit erythrocytes in *S. plicata,* are answered in Table I.

It is clear that the hemagglutinin to rabbit erythrocytes was eliminated through the absorption by mouse erythrocytes in *S. plicata* and the hemagglutinin to rabbit erythrocytes of *H. hilgendorfi* was also eliminated through the absorption by rat erythrocytes. Conversely, the absorption by rabbit erythrocytes eliminated the hemagglutinating activity to mouse erythrocytes in *S. plicata* and to rat erythrocytes in *H. hilgendorfi*. The hemagglutinin of each species of ascidian is thought to be homogeneous. However, the hemagglutinin of the two species of ascidians are not the same because the coelomic fluid of *S. plicata* could not aggregate rat erythrocytes which were aggregated by that of *H. hilgendorfi*. Moreover, the hemagglutinin of *S. plicata* to rabbit erythrocytes was not absorbed by rat erythrocytes and also that of *H. hilgendorfi* was not absorbed by mouse erythrocytes. Therefore, it may be assumed that the hemagglutinin of each of these two ascidians reacts to a different position (hapten) on the surface of the rabbit erythrocytes.

Chemical properties of hemagglutinin. Some chemical properties of hemagglutinin were studied by using the coelomic fluid of *Styela plicata.*

The coelomic fluid dialyzed to saline was incubated at 37° C for 5 hours in the buffer solutions of various pHs. After incubation, the reaction mixture was centrifuged, and after adjusting pH to 7.2 the supernatant was used for the hemagglutinin test. The stability of the hemagglutinin was very high in the region of neutral and alkaline pHs but low in strong acidic conditions (Table II).

The hemagglutinic activity was not affected by the overnight dialysis to physiological saline. The coelomic fluid of *S. plicata* was dialyzed to saline containing 0.01 M EDTA overnight, and its hemagglutinating activity was tested. The activity

TABLE I

Specificity of hemagglutinin. For further details, see text

Ascidian	Erythrocyte used for absorption	Titers of hemagglutinin to		
		Rabbit	Rat	Mouse
Halocynthia hilgendorfi	No absorption			
	Primary (×2)	$+10 \times 2^7$	$+10 \times 2^4$	–
	Secondary (×4)	$+10 \times 2^6$	$+10 \times 2^3$	–
	Rabbit erythrocyte			
	Primary (×2)	$+10 \times 2^3$	–	–
	Secondary (×4)	+10	–	–
	Rat erythrocyte			
	Primary (×2)	$+10 \times 2^4$	–	–
	Secondary (×4)	$+10 \times 2^1$	–	–
	Mouse erythrocyte			
	Primary (×2)	$+10 \times 2^7$	$+10 \times 2^4$	–
	Secondary (×4)	$+10 \times 2^6$	$+10 \times 2^3$	–
Styela plicata	No absorption			
	Primary (×2)	$+10 \times 2^8$	–	$+10 \times 2^1$
	Secondary (×4)	$+10 \times 2^7$	–	+10
	Rabbit erythrocyte			
	Primary (×2)	$+10 \times 2^2$	–	–
	Secondary (×4)	+10	–	–
	Mouse erythrocyte			
	Primary (×2)	$+10 \times 2^4$	–	–
	Secondary (×4)	$+10 \times 2^2$	–	–
	Rat erythrocyte			
	Primary (×2)	$+10 \times 2^8$	–	$+10 \times 2^1$
	Secondary (×4)	$+10 \times 2^7$	–	+10

TABLE II

pH stability of hemagglutinin. For further details see text.

pH	Buffer (0.05 M)	Titers of haemagglutinin
pH 1.9	KCl-HCl buffer	$+2^6$
pH 2.9	citrate buffer	$+2^8$
pH 4.3	citrate buffer	$+2^8$
pH 5.5	citrate buffer	$+2^{10}$
pH 6.0	citrate buffer	$+2^{12}$
pH 7.8	phosphate buffer	$+1\,2^{12}$
pH 8.6	borate buffer	$+1\,2^{12}$
pH 9.6	borate buffer	$+1\,2^{12}$

did not change as a result of this dialysis. Nor did the addition of $CaCl_2$ (2 mM) or $MgCl_2$ (2 mM) change the activity. Nor indeed did the addition of $CaCl_2$ (2 mM) and $MgCl_2$ (2 mM) change its activity.

The coelomic fluid was incubated at 0° C, 37° C, 75° C and 100° C for 30 minutes. No change of activity was observed at these temperatures. Moreover, the coelomic fluid was heated at 140° C for 30 minutes in an autoclave, but the hemagglutinic activity was not changed. Therefore it seems that the hemagglutinin is not protein.

The hemagglutinin was digested by trypsin in order to ascertain whether it was protein or not. The coelomic fluid of *S. plicata* was incubated with trypsin (1:250, Difco) at 37° C for several hours. Then the reaction was stopped by boiling at 100° C for 10 minutes. The reaction mixture was centrifuged at 450 × *g* for 15 minutes and the supernatant was used for hemagglutinic activity. No change of activity was observed due to these procedures.

TABLE III

Effect of periodate. For further details, see text.

Species	Periodate (M)	Titers of hemagglutinin
Halocynthia hilgendorfi	0	$+10 \times 2^8$
	0.04	—
	0.02	—
	0.004	$+10 \times 2^5$
Styela plicata	0	$+10 \times 2^6$
	0.04	—
	0.02	—
	0.004	$+10 \times 2^2$

From the evidence previously described, such as its heat stability and trypsin-resistant properties the hemagglutinin is considered to be a polysaccharide or mucopolysaccharide. To confirm this possibility, periodate treatment was performed because it is an agent known to destroy saccharide by oxidation.

The coelomic fluid was treated with various concentrations of periodate and then dialyzed to physiological saline. The reaction mixture is as follows; 1 ml of the coelomic fluid that was dialyzed to saline overnight, 0.3 ml of 0.2 M citrate buffer, pH 5.4, 0.2 ml of various concentrated periodate which was adjusted to pH 5.4 with 2 M NaOH. After incubation at 25° C for 3 hours, the reaction mixture was dialyzed to saline overnight to remove periodate. After centrifugation hemagglutinic activity of the supernatant was measured. The hemagglutinic activity was completely destroyed by 0.02 M periodate as shown in Table III. Since the hemagglutinin incubated at these pH conditions without periodate did not change its activity at 25° C, the destruction of activity must be due to oxidation of saccharide by the periodate.

The hemagglutinin was not dialyzable at 5° C overnight. Moreover, when ammonium sulfate was added to the coelomic fluid up to 50% saturation level, hemagglutinin was found in the precipitate. The precipitate was insoluble in water but was soluble in 0.85% NaCl. These properties suggested that it is a high-weight molecular substance. The rough molecular weight was estimated by the gel-filtration on Sephadex. The coelomic fluid was heated at 100° C for 15 minutes, then centrifuged to remove the precipitate and applied to Sephadex G-100 column. A typical elution pattern was shown in Figure 1. The same experiment was done using the Sephadex G-200 column. As shown in Figure 1, two peaks appeared, and hemagglutinic activity was found at both peaks when Sephadex G-200 was used. The coelomic fluid seems to contain two molecular species of hemagglutinin. Assuming that the hemagglutinin is polysaccharide, the molecular weight of the smaller one could lie between 150,000 and 800,000 and the larger one be over 800,000.

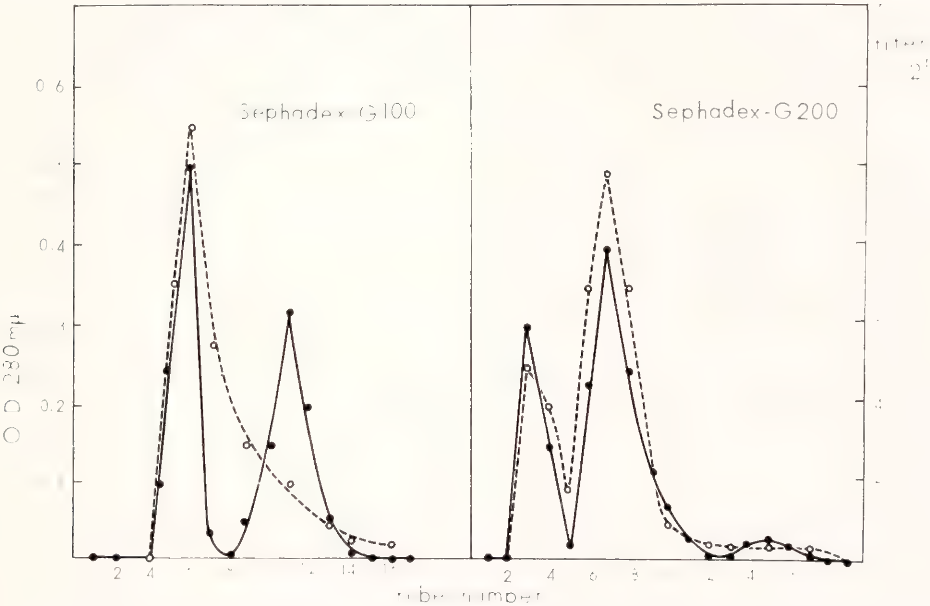

FIGURE 1. The elution pattern of the boiled coelomic fluid of *S. plicata* through sephadex G-100 and G-200 column. The coelomic fluid was boiled at 100° C for 15 minutes. After centrifugation, the supernatant ($O.D._{280\ m\mu} = 2.4$) was applied to the column. The elution buffer was phosphate buffered saline, pH 7.2 and the column size was 1.6 × 30 cm. One tube contains 6 ml of fractionated solution. Symbols used are: —●—●—, optical density at 280 mμ; - - ○ - - ○ - -, titers of hemagglutinin, 2^n.

Effect of the coelomic fluid on phagocytosis

The coelomic fluid of ascidians contained the hemagglutinin which was considered to be polysaccharide or mucopolysaccharide. It also contained a large number of coelomic cells. The fluid was examined to determine whether the ascidian's hemagglutinin could enhance phagocytosis of cells as with mammalian antibodies, although its chemical nature differed.

Identification of phagocytes in the coelomic fluid. After fixation and staining by Giemsa, the coelomic cells of *S. plicata* which have properties of adherence to the glass surface were observed. Four cell types of phagocytes were distinguished.

Fine-granular amoeboid cells were abundant and about 80% of coelomic cells are in this type. The cytoplasm contains many fine granules stained reddish with Giemsa which characterized these cells (Fig. 2, a). The size of the cells are of 5–10 μ. They show active amoeboid movement and phagocytosis (Fig. 2, e).

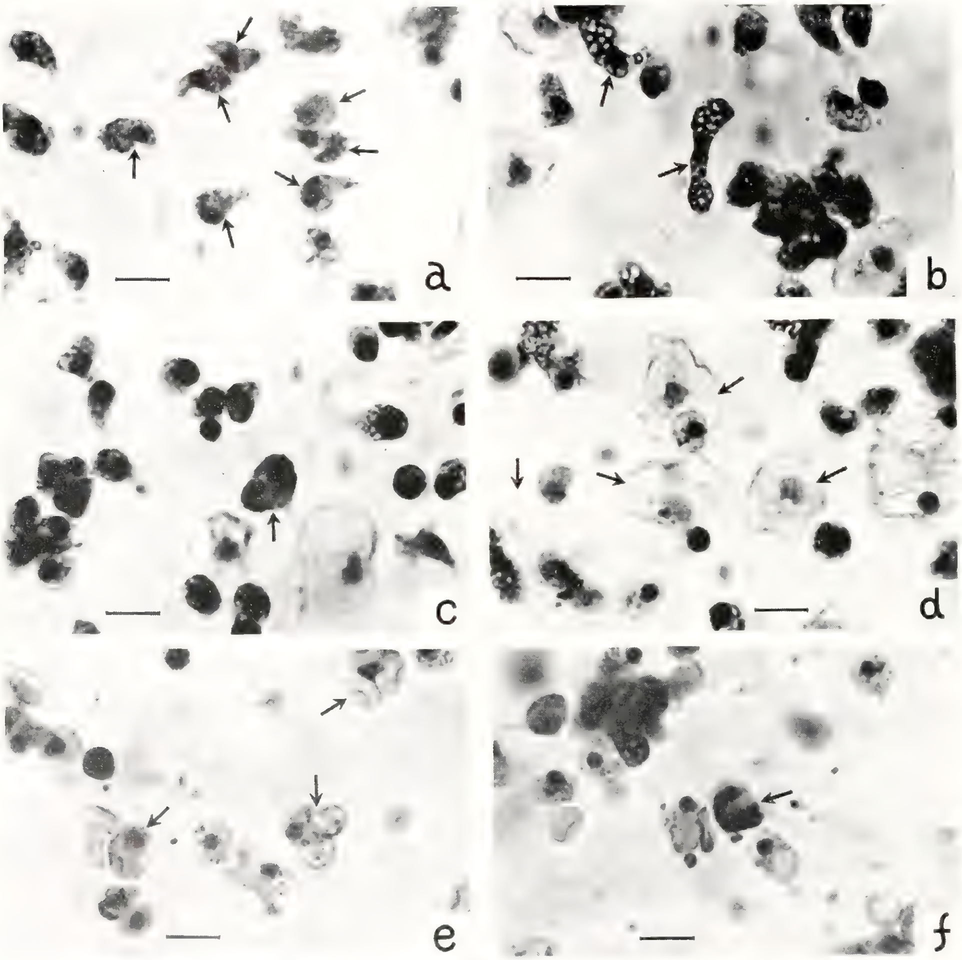

FIGURE 2. Phagocytic cells in the coelomic fluid of *Styela plicata*; (a) fine granular amoeboid cells; (b) granular amoeboid cells; (c) large basophilic cells; (d) vacuolated cells; (e) fine granular amoeboid cells which took in rabbit erythrocytes; (f) phagocytosis by granular amoeboid cell. The scale line indicates 10 μ.

The cells tend to aggregate when they come in contact with air. And when a toxic dose of dye or erythrocytes was injected into the coelomic cavity, these cells were also observed to aggregate in a sheet.

Granular amoeboid cells form about 15% of coelomic cells. They are large granules which are vitally stained with Nile blue. They showed a very active amoeboid movement and very elongated forms were frequently observed in a smear preparation (Fig. 2, b). They showed phagocytosis (Fig. 2, f).

Large basophilic cells are difficult to find because they are about 1–2% of the total number of cells. The cell size was about 10–15 μ. The cytoplasm was stained with a characteristic grayish blue. One or two vacuoles were usually observed. The nucleus was relatively small and round, and had an eccentric position (Fig. 2, c). Cells usually do not contain erythroyctes, but phagocytosis was occasionally observed.

Vacuolated cells are thin and elongated and have several vacuoles (Fig. 2, d). Phagocytic activity of these cells was very evident. The number of the cells varied depending on physiological conditions, for example, the cell number increased after starvation.

Effect of the coelomic fluid on phagocytosis. A test was made to determine whether the phagocytosis of the coelomic cells of *S. plicata* was affected by the presence of the coelomic fluid which contained the hemagglutinin.

TABLE IV

The Effect of the coelomic fluid on phagocytosis. For further details see text.

The concentration of coelomic fluid	% of phagocytic cells	s.e.m.
Sea water	49%	±1.8
$\frac{1}{5}$ coelomic fluid + $\frac{4}{5}$ s.w.	50%	±3.2
$\frac{1}{3}$ coelomic fluid + $\frac{2}{3}$ s.w.	48%	±1.8
$\frac{1}{2}$ coelomic fluid + $\frac{1}{2}$ s.w.	50%	±3.5
Coelomic fluid	49.5%	±1.7

The number of cells per milliliter of the coelomic fluid was estimated by haemocytometer. Some variations were observed between the different animals. The mean value of ten animals was 7.5×10^6 and the standard error of mean was ± 2.0.

One milliliter of coelomic fluid was poured on the dishes ($d = 3.2$ cm) and after ten minutes setting, the coelomic fluid was pooled and centrifuged at 450 × g. The supernatant was diluted with sea water at various concentrations ($\frac{1}{2}$ coelomic fluid + $\frac{1}{2}$ sea water; $\frac{1}{3}$ coelomic fluid + $\frac{2}{3}$ sea water; $\frac{1}{5}$ coelomic fluid + $\frac{4}{5}$ sea water) and added to the dishes. Fixed rabbit erythrocytes were added and after phagocytosis had proceeded for 20 minutes, the cells were fixed. The percentage of viable cells were determined by staining with 0.04% nigrocine. It showed over 95% after setting for 30 minutes. The number of coelomic cells which had taken an erythrocyte into their cytoplasm was counted. One ml of the coelomic fluid which contained the cells (7.0×10^6) was poured in to a plastic dish ($d = 3.2$ mm). 0.1 ml of red blood cells (1.4×10^6) was added. Twenty minutes later, cells were fixed and stained by Giemsa. $125 \times 125\ \mu^2$ each of phagocytic cells and non-phagocytic cells were counted. There were almost 50 cells in total per $125 \times 125\ \mu^2$. The cells were counted seven times. The results are shown in Table IV. It seems that the coelomic fluid was not essential for phagocytosis. To determine the effect of the coelomic fluid on phagocytosis more decisively, an experiment using the anti-coelomic fluid antibody is in progress.

However, from microscopical observations, it seemed that the coelomic cells aggregated with erythrocytes and with each other more actively in the presence of the coelomic fluid than in its absence. It also seemed that the coelomic fluid enhanced the adhesion of cells to glass surfaces.

DISCUSSION

Present results showed that two ascidians possessed hemagglutinins for several mammalian erythrocytes in their coelomic fluids. These hemagglutinins are large molecules which can be absorbed by erythrocytes. These properties are similar to those of isohemagglutinin present in mammalian serum. But the hemagglutinin of ascidians is thought to be polysaccharide or mucopolysaccharide because it is very heat stable and destroyed by periodate.

The chemical properties of the hemagglutinin of ascidians are different from those of other invertebrates. Mackay *et al.* (1969) reported recently that the hemagglutinin of a crayfish was a protein which enhanced adhesion and phagocytosis of red cells by the phagocytic cells. The hemagglutinin of oysters was also reported as proteinaceous by Acton *et al.* (1969). The hemagglutinin of ascidians is similar to plant hemagglutinins rather than those of animals (Aub, *et al.*, 1963).

Burnet (1968) has recently suggested that such hemagglutinins may be forerunners of vertebrate immunoglobulins. However, the hemagglutinin of ascidians is not chemically related to vertebrate immunoglobulins. Moreover, structurally the hemagglutinin of oysters is demonstrably different from mammalian immunoglobulin (Acton *et al.*, 1969). The body fluids of ascidians possess heat labile bacterioagglutinin (unpublished data of the authors). There remains the possibility that such a protein in the coelomic fluid (other than hemagglutinin) is the ancestral precursor of immunoglobulin.

The biological functions of the hemagglutinin in ascidians still remain obscure, and more detailed experiments are required. The hemagglutinin is not essential for phagocytosis by coelomic cells in this experimental system, nor was it found that the hemagglutinin activated phagocytosis. However, this does not exclude the possibility that the hemagglutinin which became bonded to the cell surface of phagocytes could play an important role in the discrimination between self and not-self. An experiment using the anti-hemagglutinin is now in progress and should clarify the problem.

SUMMARY

The occurrence of a natural hemagglutinin in the coelomic fluid of solitary ascidians, *Styela plicata* and *Halocynthia hilgendorfi* is reported. The hemagglutinin aggregated some mammalian erythrocytes and was absorbed by them. The hemagglutinins of the two ascidian species are specifically distinct.

The hemagglutinin of *Styela plicata* is a large molecule which is very heat stable, resistant to trypsin digestion, but is destroyed by periodate. These data suggest that the hemagglutinin is polysaccharide or mucopolysaccharide.

The hemagglutinin has no apparent opsonic effect, but it seems to play a role adherence of cell-to-cell and cell-to-glass surface.

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DIMENSIONS AND ULTRASTRUCTURE OF TOADFISH GILLS

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During the last 20 years the gill areas of many fish species have been measured and a fairly comprehensive impression gained of the overall range among fishes belonging to different groups and having a variety of life habits. It has become clear that more detailed knowledge is now required for individual species and especially in combination with studies on the ultrastructure of the gills and physiology of gas exchange. Of the marine fishes whose gill areas have been measured, the toadfish (*Opsanus tau*) is of particular interest because of the relatively low value found for its gill area (Gray, 1954). This seems to be correlated with the sluggish habits of this species and other aspects of its physiology such as its respiratory dependence down to very low O_2 tension in water (Hall, 1929). This paper is concerned with the fine structure of the toadfish gills together with a more extended analysis of the data summarized previously (Gray, 1954).

MATERIALS AND METHODS

The toadfish ranged in size from 15–800 g; the gills were removed, fixed, and measurements made according to the method described by Gray (1954). This involved the counting of filaments of the three gill arches, sampling of secondary lamellae and measurement of their areas. The fish were obtained from Woods Hole, Massachusetts, and Beaufort, North Carolina. No significant difference was observed between these two populations. These measurements were analyzed for the relationship between body weight and different components of the gill area using the method of linear logarithmic transformation as described by Muir and Hughes (1969). Regression lines were fitted using the method of least squares with Wang and Olivetti computers. Other toadfish gills were fixed at Beaufort for inspection of their secondary lamellae, and for electron microscopy after fixation in 2.5% glutaraldehyde in cacodylate buffer (pH 7.2) with subsequent postfixation in 1% osmium tetroxide and embedded in Vestopal. The material was examined under AEI 6G and Phillips 200 and 300 electron microscopes.

RESULTS

Gross Morphology

Toadfish gills are reduced relative to those of most fish for there are only three holobranchs on each side. The filaments are widely-spaced along the gill arches and this relative coarseness of the sieve extends to the secondary lamellae as there are only 10–13/mm on each side of a gill filament (Gray, 1954).

A detailed analysis of a single specimen (400 g) showed that the average number of secondary lamellae/mm was 11.0, 12.7 and 11.6 for the tip, middle and base of a filament. The filaments of the posterior hemibranch are longer than the anterior filaments for the first and second arches, but the anterior filaments are longer on the third arch (Fig. 1).

Total gill area and body weight. The relationship between total gill area and body weight for 58 specimens that had been measured, is plotted on log/log coordinates in Figure 2. It is clear that the gill area increases with increasing size, the regression line obeying the equation

$$\text{Log } A = 560.7 + 0.79 \log W$$
$$(i.e., A = 560.7\ W^{0.79})$$

Correspondingly the regression line for gill area/g against body weight has a slope of − 0.217. A summary of the gill area data divided into 50 g classes is given in Table I. The slope of the regression line relating these average areas to body weight is 0.779.

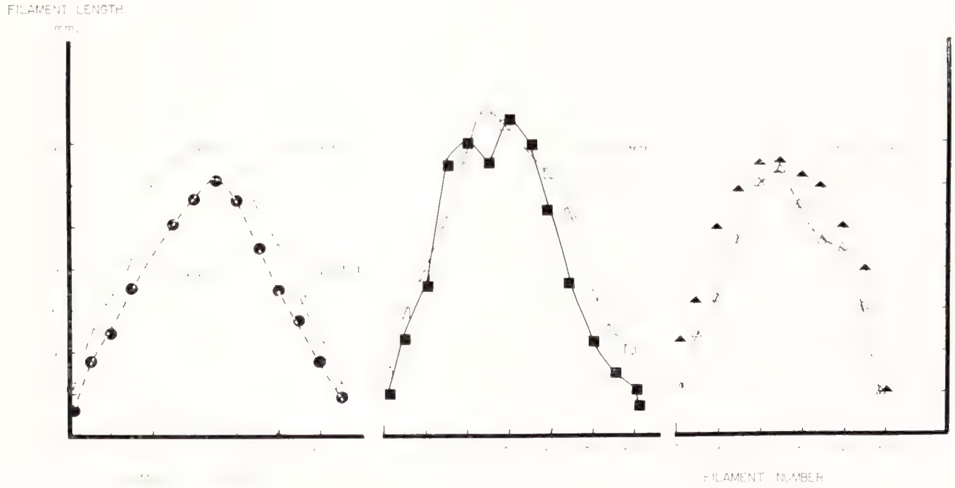


FIGURE 1. Graphs to show the length of filaments on the different gill arches of the left side of a toadfish (*ca.* 400 g). Solid symbols indicate the lengths of filaments of the anterior hemibranches in each case. On average, every 5th filament was measured.

Components of the gill area. When the measurements for gill area determinations are examined, it is clear that the increased area of larger fish is mainly due to a greater total number of secondary lamellae (Fig. 3 C). The number of gill filaments increases rapidly at body weights up to 50 g, but above that size there is relatively little increase in the total number of filaments (N). Filament length increases continuously and consequently the number of secondary lamellae (Fig. 3). This is apparent when the data are plotted on log/log coordinates showing that the regression line for an increase in number of secondary lamellae has a slope of 0.42 whereas that for filament number increases as $W^{0.087}$. Another important factor is the increased area of the secondary lamellae themselves, as indicated below.

The secondary lamellae are relatively large in the toadfish, and as in other species their shape varies according to their position on the gill arches and espe-

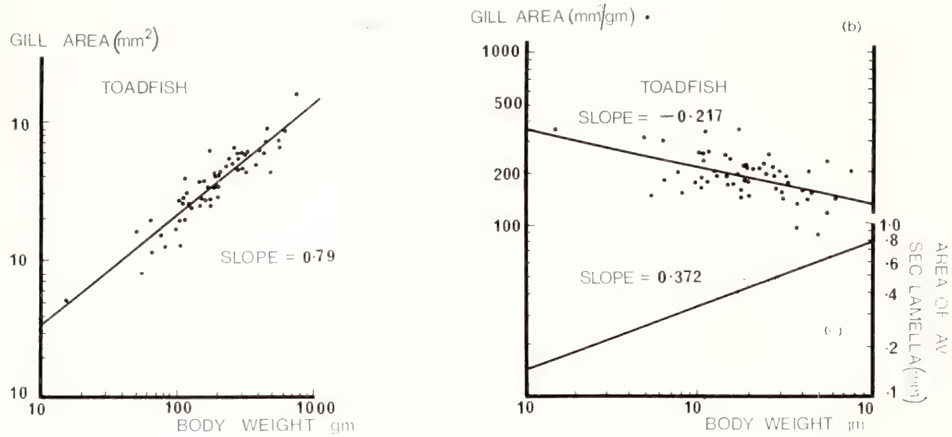


FIGURE 2. Relationships between areas of the gill system and body weight plotted on log/log coordinates: (a) relationship between total gill area and body weight; (b) gill area/g and body weight; (c) the unweighted average area of a secondary lamella and body weight.

cially on the gill filament (Fig. 4). Variations in shape and area of the secondary lamellae from different parts of the gill are related to the flows of water and blood and their role in gas exchange, about which little is known for toadfish. Secondary lamella areas can be plotted out in different ways, indicating their increasing area in the direction of water flow (Fig. 4). Such ways of summarizing the form of

TABLE I

Average weights and gill areas for 50 g classes of 58 specimens of toadfish. Averages for fish within the same order of magnitude are also given

Wt. class	Average wt.		Gill area	
	g	No.	mm²/fish	mm²/g
0–50 g	15	(1)	5,236	349.07
50–100	69.5	(7)	14,199	204.3
100–150	120.4	(13)	26,217	217.75
150–200	182	(13)	35,248	193.67
200–250	232	(4)	48,737	210.07
250–300	277.6	(5)	56,906	204.99
300–350	317.5	(4)	52,652	165.83
350–400	374	(2)	42,168	112.75
400–450	425	(3)	71,529	168.30
450–500	471	(2)	56,977	120.97
500–550		(0)		
550–600	560	(2)	69,207	123.58
600–650	620	(1)	86,867	140.11
650–750		(0)		
750–800	776	(1)	160,362	206.65
10–100	62.7	(8)		228.75
100–1000	260	(50)		191.46

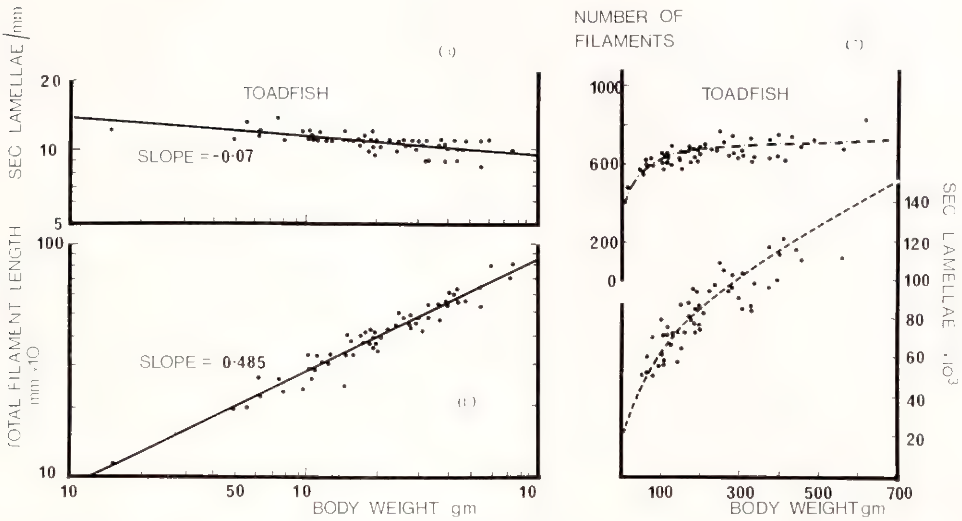


FIGURE 3. Graphs to show (a) relationship between average number of secondary lamella/mm on one side of a filament and body weight plotted on log/log coordinates; (b) log/log plot of the total filament length against body weight and (c) the increase in total number of gill filaments and secondary lamellae (dotted line) of 58 toadfish.

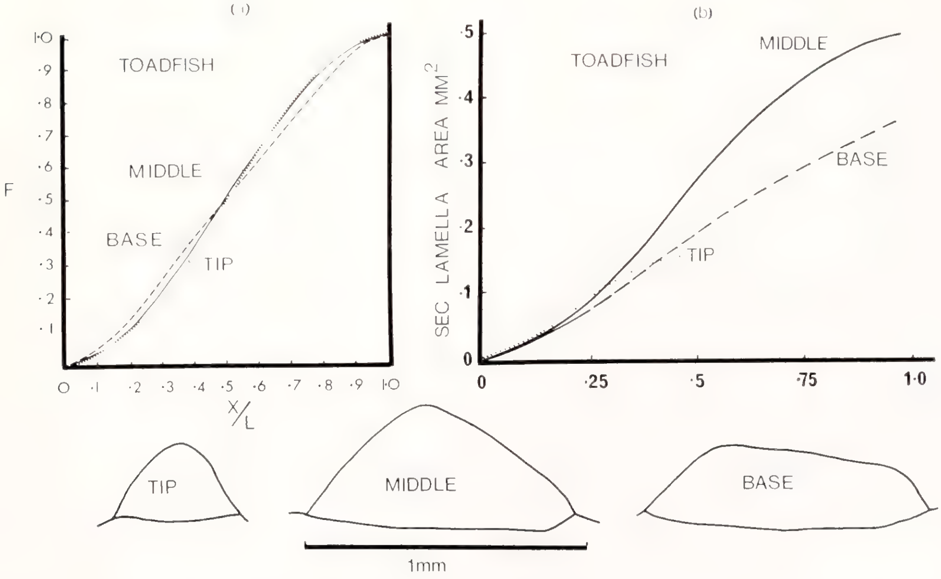


FIGURE 4. Outline shapes of the secondary lamellae from the tip, middle and base of filament 45 of arch 2 of a 400 g toadfish. The change in area of the secondary lamellae in the direction of water flow are plotted (a) as fractional cumulative areas (F) with respect to the fractional path length (X/L), (b) as cumulative areas (mm²) along the length of the secondary lamella in the direction of water flow.

TABLE II

Opsanus tau. *Results of regression analysis for the gill area and its component parameters.*

	W = 1 g			W = 10 g		
		Limits			Limits	
		95% Conf	Tol		95% Conf	Tol
Total fil length (mm)	301.2	363.3	394.4	923.7	1028	1150
		250.9	231.4		830.7	741.8
No. sec. lam./mm on one side of filament	15.86	18.33	19.53	13.37	14.53	15.87
		13.71	12.87		12.30	11.27
Ave. area of sec. lam. (mm²)	0.06037	0.09781	0.1206	0.1412	0.1866	0.2502
		0.03726	0.03021		0.1082	0.08074
Total area (mm²)	560.7	921.9	1144	3459	4599	6024
		341.0	274.9		2604	1929
Wt. specific area (mm²/g)	585.6	934.2	1050	355.4	462.9	551.2
		367.1	326.6		272.9	229.2

a secondary lamella are of value in analyses of the O_2 tension gradients along the secondary lamella (Hughes and Hills, 1971; Hughes, 1972a, 1972b). In Gray's (1954) original data, secondary lamellae were termed "lamellae" or "platelets" and refer to the *two* secondary lamellae which are more or less opposite at a given level on a gill filament. Hence for comparison with data for other species it is usually necessary to halve his figures for areas of individual secondary lamellae and to double the total numbers of secondary lamellae.

The increase in total number of secondary lamellae with body weight is largely due to the increased length of the gill filaments, for the spacing (1/d′) remains relatively constant. The data for total filament length and number of secondary lamellae/mm are plotted on log/log coordinates in Figure 3 A and B and the relevant statistical information is given in Table II. Thus:

Secondary lamellae/mm on one side of filament $= 15.86\ W^{0.074}$; and
Total filament length (mm) $= 302.1\ W^{0.485}$

Fine structure of the toadfish secondary lamella

The structure of the secondary lamella as seen under the electron microscope is similar to that of other teleost fish (Fig. 5) (Hughes and Grimstone, 1965; Newstead, 1967; Hughes and Wright, 1970; Tovell, Morgan and Hughes, 1970). The outer epithelial layers are separated from the pillar cell flanges by a well-marked basement membrane which consists of three well-defined layers: (i) an outer clear layer, (ii) a fine fibrous layer, followed on the inner side by (iii) a much thicker collagenous layer being about 4 or 5 times the thickness of the other two layers, which together constitute the basal lamina (Figs. 6, 8). In some places the outer homogeneous layer seems to have protuberances into the inner border of the epithelial layers. The collagen fibrils have clearly defined striations,

TABLE II

Values for 1, 10, 100, and 1000 g fish are given, together with the confidence and tolerance limits

W = 100 g			W = 1000 g			$Y = aW^b$			
	Limits			Limits					
	95% Conf	Tol		95% Conf	Tol	b	S_b	a	S_a
2825	2920	3433	8638	9191	10570	0.4854	0.0174	302.1	1.097
	2732	2325		8117	7060				
11.27	11.51	13.12	9.51	9.98	11.13	−0.0740	0.0137	15.86	1.075
	10.98	9.69		0.96	8.13				
0.3347	0.3624	0.5529	0.7879	0.9301	1.1329	0.372	0.046	0.0604	1.274
	0.3090	0.2026		0.6676	0.4672				
21350	23330	35850	131700	156500	225800	0.790	0.047	560.7	1.282
	19530	12720		110900	76850				
215.7	230.1	308.0	130.9	152.4	191.8	−0.217	0.045	585.6	1.265
	202.1	151.0		112.4	89.32				

repeating every 640 Å. The collagen layer is particularly thick next to each pillar cell body where it gives rise to the columns.

Because of its thick collagen layer, the toadfish gill is especially suitable for inspection of the structure of the columns which, as in other species, are extracellular. The number of columns/pillar cell is about five. The not uncommon folding observed in the pillar cell columns (Fig. 6) perhaps suggests a contracted condition of these cells at fixation. In both transverse and longitudinal sections of the pillar cells, it is apparent that the columns also contain a type of fine fibril which is not, however, a direct continuation of the fine fibrous layer of the basal lamina (Fig. 6). In the pillar cell flanges, fibrils appear in cross-section which are very suggestive of collagen. Another interesting feature observed in toadfish pillar cells is the presence of cytoplasmic processes jutting into the blood channel from the main cell body or its flanged part. These processes sometimes contain what appear to be collagenous fibrils. The pillar cell body contains many types of granule and is well provided with cytoplasmic filaments suggestive of contractile protein, particularly in the neighborhood of the columns. There is also some evidence of such filaments in the endothelial cells which line the marginal channel of the secondary lamella. Typical endothelial granules (Weibel and Palade, 1964; Hughes and Wright, 1970; Weibel and Hughes, in preparation) were specially prominent in the toadfish and their presence in these cells only and not in the pillar cell flanges was very clearly defined in most cases. In addition, the endothelial cells have many pinocytotic vesicles and specially large ones are often seen bordering the collagen layer of the basement membrane. Unlike the comparable layer of the mammalian lung, this endothelium has no underlying basal lamina.

The outer epithelial layers have a number of points of interest. Microvilli are not very obvious in most sections but there seems to be some surface sculpturing as there are deep invaginations between epithelial cells, especially in the crypts

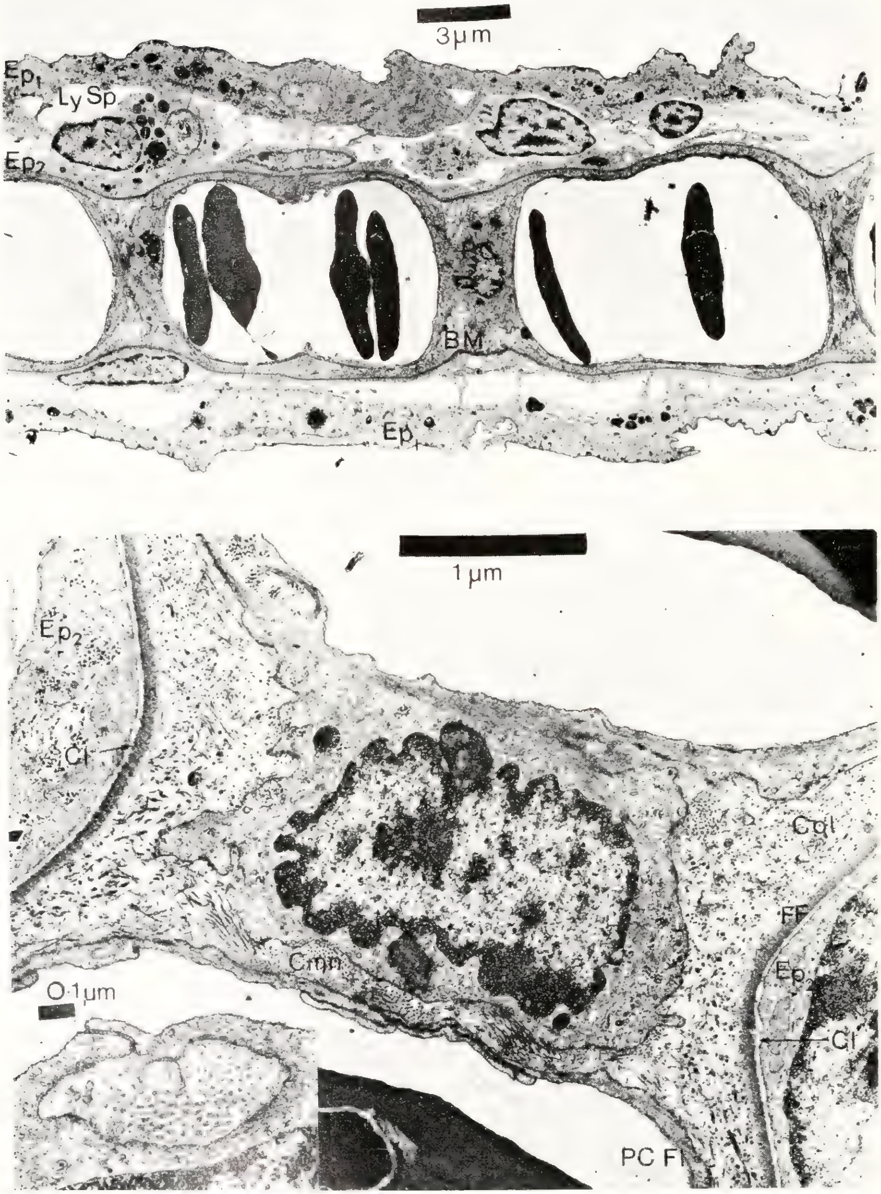


FIGURE 5. *Opsanus tau.* Electron micrograph to show the basic structure of a secondary lamella cut transverse to the direction of blood flow. Three pillar cells (PC) are visible separating and lining the blood channels. Notice the sculpturing of the outer epithelial layer (Ep_1) and the different types of lymphocyte (monocytes and macrophages) to be found in the lymphoid space (Ly Sp), between the two epithelial layers. The basement membrane (BM) separates the inner epithelial layer (Ep_2) from the pillar cells.

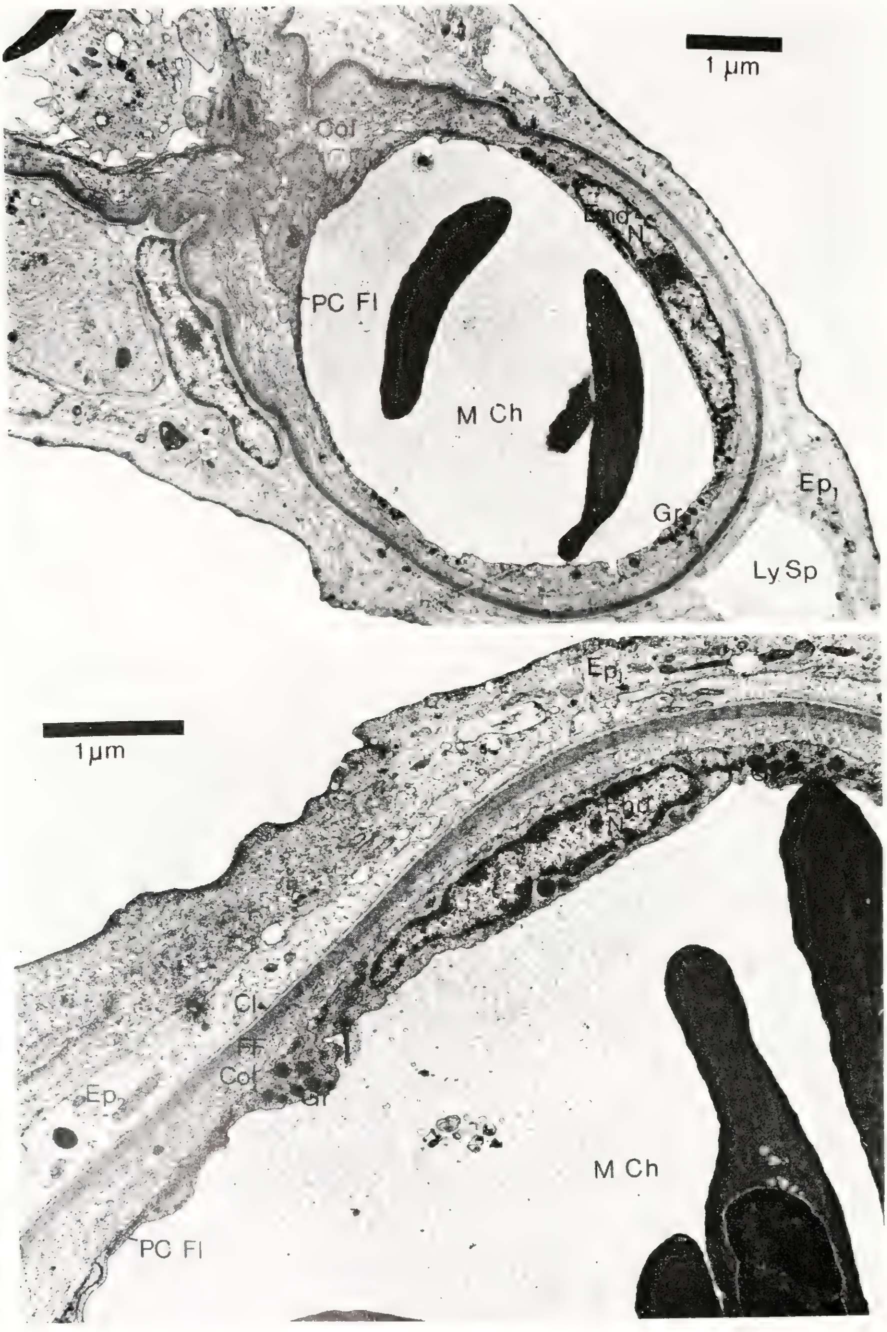
between secondary lamellae. Many fine-folded junctions are found between cells of the outer epithelial layer and where these come to the surface they sometimes resemble microvilli similar to those noticed in the pollack between adjacent epithelial cells. Desmosomes are also visible at these junctions. Epithelial cells in the region outside the marginal channel are often particularly thick (Fig. 7). Frequently the outer layer of epithelial cells seems to form a continuous coat which may be separate to some extent from the underlying epithelial cells. These "lymphoid" spaces often contain a number of lymphocytes of different types but the space clearly does not contain plasma. Transitional stages from monocytes to macrophages are often visible (Fig. 5). Amoebocytes are also present in the blood channels (Fig. 7), but no connection has been established between these channels and the intra-epithelial spaces. The outer surface of the epithelial cells is more darkly staining than the rest. Chloride cells are common, both in the crypts and other regions of the secondary lamella and often seem to be separated from the outer surface by the outer epithelial layer. The cytoplasm of the chloride cells is also somewhat unusual.

The water/blood pathway is relatively thick in certain parts of the sections, but in others it may be as thin as 3 μm. The epithelial layers usually constitute from $\frac{1}{2}$ to $\frac{3}{4}$ of the total water/blood distance. The flange and endothelial layers are very thin and, as mentioned above, the basement membrane and particularly its collagen layer, is noticeably thickened in this species. The collagen layer is at least 1 μm thick.

Discussion

The toadfish data analyzed in this paper are more extensive than that available so far for adults of any other marine species. The results obtained are in substantial agreement with those given for tunas (Muir and Hughes, 1969) with which they contrast considerably because of the great differences in activity of the two species. Some of the most obvious differences in gill dimensions are shown in Table III which summarizes the "a" and "b" values in the relationship $Y = aW^b$, where Y is the gill area or its constituent parameters. Clearly the slope of the log/log regression line for total gill area of tunas is greater than that of toadfish and similar differences are seen in the component parameters; for both fish they are all of the same order of magnitude. Far greater differences are observed in the "a" or intercept values, *e.g.*, that for total area of tuna is 6 times that of toadfish. This is made up of a 16-fold difference in the figure for total filament length and a four-times greater number of secondary lamellae/mm. However, for the area of an average secondary lamella the "a" value for toadfish is about 14 times greater than for tuna. These differences clearly support the generalization that more active fish tend to have a greater number of closely-spaced secondary lamellae which are of relatively smaller area (Hughes, 1966). Figures for the dolphin

FIGURE 6. A single pillar cell from a secondary lamella of the toadfish showing its flange (PC Fl) lining the blood spaces, the thickened collagen layer (Col) of the basement membrane, and a single column (Cmn) in longitudinal section. The fine fibrous (FF) and outer clear (Cl arrow) layers of the basal lamina can easily be distinguished. A transverse section across a single column is shown at higher magnification as an insert, bottom left.


1 μm
Col
End c
N
PC Fl
M Ch
Gr
Ep$_1$
Ly Sp
Ep$_1$
1 μm
End
N
Cl
Col
Gr
Ep$_2$
M Ch
PC Fl

FIGURES 7–8.

Table III

Comparison of the intercept (a) and slope (b) of the regression lines for the components of the gill areas of toadfish, Coryphaena *and tunas**

		Toadfish		Coryphaena		Tunas	
		a	b	a	b	a	b
Total filament length (mm)	L**	302.1	0.485	1879	0.431	5594	0.382
Secondary lamellae/mm on one side of filament	$\frac{1}{d'}$	15.96	−0.079	33.81	−0.036	60.87	−0.089
Average area of sec. lam. (mm^2)	bl	0.0604	0.372	0.0377	0.327	0.0046	0.583
Total area (mm^2)	A	560.7	0.79	5208	0.713	3151	0.875

* Based on Muir and Hughes (1969).

** Symbols as in Hughes (1966).

fish, *Coryphaena* (Table III) are closer to tuna, and data for other fish fall between the toadfish and *Coryphaena.*

From the respiratory point of view, a very important parameter is the distance (t) separating the blood and water which is substantially greater in the toadfish than in the tuna. The general relationship between O_2 consumption ($\dot{V}_{O_2}$) and area of the respiratory surfaces (A) is given in the equation:

$$\dot{V}_{O_2} = \frac{KA\ \Delta P_{O_2}}{t}$$

which may be rearranged:

$$\frac{\dot{V}_{O_2}}{\Delta P_{O_2}} = K \cdot \frac{A}{t} = D_t,$$

the diffusing capacity of the tissue barrier of the gills (Hughes, 1972b). K is the permeation constant of Krogh expressed as ml $O_2/\mu m/cm^2/mm$ Hg and is usually assumed to be the same for the different layers of the water/blood barrier which has an overall thickness *t*. However, if there are marked differences in K between these layers then the much greater thickness of the collagen layer in toadfish could be significant.

The available figures give estimates for gill area of a typical 100 g toadfish of about 21,000 mm^2 whereas that of a bluefin tuna of the same size would be of the order of 200,000 mm^2. The water/blood distances are approximately 5 μm for

Figure 7. Section through the marginal channel of a toadfish secondary lamella. The nucleus (End N) of an endothelial cell is clearly visible but the section only passes through the edge of a pillar cell of the outer row. Bordering the latter a greatly thickened collagen layer (Col) can be seen. The epithelial layers along the outer edge of the marginal channel (M Ch) are thickened and separated by lymphoid spaces (Ly Sp), so that the water/blood distance is much greater here than on the lateral aspects of this channel.

Figure 8. Higher magnification electronmicrograph of a part of the marginal channel (M Ch) to show the different components of the water/blood barrier. The section passes through a flattened endothelial nucleus (End N) and typical granules (Gr) are visible in this cell which clearly differentiate its cytoplasm from that of the pillar cell flanges (PC Fl) which line most of the blood channels.

toadfish and 0.5 μm in tunas (Hughes, 1970). Consequently the diffusing capacities (D_t) are about 42 K and 4000 K, respectively. Thus the same differences in O_2 tension across the gills would result in the transfer of an amount of O_2 that is about 100 times greater for the tuna than a toadfish. This example clearly emphasizes the importance of the differences in area and thickness of the two species, but as yet no physiological measurements have been made of the diffusing capacity of the gills (D_g) in these two species so that these estimates must remain anatomically-based.

Ultrastructural studies of the toadfish gill have also served to emphasize one or two interesting features which are probably general for most fish gills.

As mentioned previously, the collagen layer of the basement membrane is particularly well developed in this fish and emphasizes its importance as a supporting structure in these relatively coarse gills, which may continue to function when the fish is out of water. Two epithelial layers are present as in most other species, but in the toadfish the space observed between these two layers is particularly noticeable. The presence of macrophages and other leucocytes in the lymphoid space suggests a protective function, perhaps analogous to the alveolar macrophage of the mammalian lung. Clearly the respiratory surface of the fish being constantly ventilated by water could not have macrophages on its outer surface. Thus the presence of such cells between the two epithelial layers can be related to the difference in respiratory medium. This space has generally been omitted in discussions of the water/blood barrier of fish. If it is taken into account, the constituent layers can be listed as follows (1) outer epithelial layer; (2) lymphoid space; (3) inner epithelial layer; (4) basal lamina; (5) collagen layer; (6) pillar cell flange—instead of epithelium (1, 2, 3), basement membrane (4 and 5) and pillar cell flange (6).

Comparison of the fine structure of the toadfish secondary lamella with that of tuna is also instructive and emphasizes the correlation between structure of the respiratory surface and the habits of the animals.

We wish to thank Knut Schmidt-Nielsen and Vance Tucker for providing and assisting with computer facilities. Analysis of this data was begun during a visit of G. M. H. to the Department of Zoology at Duke University, N. Carolina, and both there and at Beaufort he enjoyed excellent hospitality.

SUMMARY

1. This analysis of the measurement from the gills of about 60 toadfish using log/log transformation has shown that the gill area and its constituent parts increases with body weight as follows:

Total area (mm^2) = 560.7 $W^{0.79}$
Total filament length (mm) = 302.1 $W^{0.485}$
Secondary lamellae/mm on one side of filament = 15.99 $W^{-0.075}$
Average bilateral area of a secondary lamella (mm^2) = 0.06 $W^{0.372}$

2. A comparison with the corresponding data for other fish, particularly tunas, shows differences in the values of these relationships and confirms the general

conclusion that the gills of more sluggish fish have a smaller number of larger secondary lamellae and relatively wider spaces through which the water flows.

3. An electron microscope study of the toadfish secondary lamella has shown the same basic structure as in other teleost fish but the collagen layer of the basement membrane is especially thick. Also noticeable are distinct lymphoid spaces between the two epithelial layers and the presence of stages in the development of cells concerned with the protection of these layers. The water/blood barrier therefore comprises the following layers in certain regions: (1) outer epithelial layer, (2) lymphoid spaces, (3) inner epithelial layer, (4) basal lamina, composed of outer clear and inner fine fibrous layers, (5) collagen layer, (6) pillar cell flange.

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A HIERARCHY OF HISTO-INCOMPATIBILITY IN *HYDRACTINIA ECHINATA*[1]

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There is a striking consistency in the biochemistry and ultrastructural morphology of all living cells. These cells do, however, recognize differences among themselves and react accordingly. Cellular recognition mechanisms are operative in dissociated embryonic cells derived from different organisms (chick and mouse), so that cells of like function remain together in chimeric aggregates, while those derived from different organs segregate (from each other) (Moscona, 1957). Dissociated sponge cells segregate according to species (Humphreys, 1963), and cells from different tissues of the same organism segregate from each other within the initial reaggregate mass (Steinberg, 1962a, 1962b, 1963). Mechanistic explanations of cellular segregation focus on differences in cellular adhesiveness (Townes and Holtfreter, 1955) due to stereospecificity of binding sites; species specific extracellular binding molecules (Humphreys, 1963); or variations in thermodynamic energies of adhesion (Steinberg, 1962a); or specific recognition sites on cell membranes for specific histocompatibility antigens (Burnet, 1970).

Recognition and interaction exist between unlike cells as well. Endocrine secretions affect specific target organs. All inductive processes involve molecular mediators. Normal development is a well integrated temporal series of inductive interactions in which one tissue chemically initiates change in a second tissue. All differentiation and morphogenesis is the result of delicately balanced intra- and and intercellular stimulation and feedback control systems. Occasionally there is a breakdown in the system, resulting in hyperplastic or neoplastic growth.

The study of developmental deviations in simple organisms may reveal the mechanisms of similar imbalances in more complex species. The Coelenterata offer a simple system in which to approach these developmental mechanisms.

The main focus of this study is the "overgrowth" phenomenon, a hyperplastic development of stolons resulting from a histo-incompatibility among genetically different strains of *Hydractinia echinata* isolated from nature. Some attention will also be given to some aspects of normal development which have been misinterpreted in the literature.

MATERIALS AND METHODS

Hydractinia echinata is an encrusting, colonial marine hydroid usually found on gastropod shells that have been appropriated by the hermit crab *Pagurus*. All

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[1] This investigation was supported in part by National Institutes of Health Grant GM 11555 and a grant from the Indiana University Foundation.

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strains used in this study were isolated from shells collected in the Woods Hole, Massachusetts area.

A hierarchy of overgrowth potential was established twice, using two sets of ten strains each. The first group of animals (Group 1; 1–5 male, 6–10 female) was started from animals scraped from the surface of shells collected in August at the time when the colonies were in full sexuality. The second group (Group 11; 1–5 male, 6–100 female) was collected in December when the colonies showed juvenile or regressive sexual development. Clones derived from the mating of these colonies were designated by the number of both parents (*i.e.,* colony 8 mated to colony 4 produced 8–4 offspring). In the F_1 generation, the overgrowers were designated 8–4(0) and their overgrown siblings were labelled 8–4(X).

Stock strains were started by scraping a piece of mat from a colony growing on a hermit crab shell. One or two feeding hydranths were then teased from this mat and their proximal, cut ends held in close contact with a glass slide by a single loop of thread tied around the slide. Within 24 hours in the faster growing strains, stolons grew out of the cut ends, adhered to the glass and thereby held the feeding hydranths to the slide. The thread was then removed. Three to five colonies were started under each thread. Stolons grew out from the transplanted hydranths so that they eventually met stolons of the other transplants. If the colonies were of the same stock, they fused, forming a continuous colony across the width of the slide. If the transplants were of different stocks, hyperplastic stolons were produced by one or both of the colonies.

The first group of experimental animals was raised on glass slides, held vertically in glass staining racks in standard ($2\frac{1}{2}'' \times 3'' \times 3''$) staining dishes. The dishes were placed on a slow, gentle horizontal shaker. The second group of animals was grown on slides in staining racks that were placed in a plexiglass tank $3'' \times 15'' \times 3''$. Water was circulated by a vibropump and passed through glass wool and charcoal filters.

The hydroids were kept in natural sea water that was pasteurized by heating to 80° C on two consecutive days and aerated for 30 minutes before use. The water was changed every 7 days. Temperatures ranged between 19° and 22° C during the course of the study. The cultures were fed freshly hatched brine shrimp (*Artemia salina*) once a day.

Precautions were taken to keep the cultures as "clean" as possible, but due to their living food and their initial isolation directly from nature, the cultures were occasionally infected by bacteria, algae and ciliated protozoa. The contaminants were kept in check by periodic light swabbing of the slides and colonies with cotton wound on a thin glass rod. Heavy infestations of ciliates were treated by placing the cultures in a dilute (100 units/ml) solution of Mycostatin for 20 minutes, which cleared the slide of surface ciliates without apparent harm to the hydroids. Heavily infected areas of the colonial mat were cut away and the excised tissue was quickly replaced by healthy new tissue.

In order to test strain compatibility, two strains were placed on a slide and allowed to grow until the stolons contacted each other. All possible binary combinations (45) of the ten strains in Group I and Group II were tested. Compatibility was also tested on gastropod shells to insure that the overgrowth phenomenon obtained in nature as well as in the laboratory. Hermit crabs were removed from their shells and kept in isolation. Hydranths of the strains to be tested were tied in place. When the colonies appeared fastened to the surface by stolonic growth, the threads were cut and the crabs were allowed back into their shells. Each crab was kept in its individual finger bowl to avoid abrasive contact with other crabs.

Vital staining was accomplished by feeding stained *Artemia* nauplii to the colony to be dyed. One drop of either 0.1% aqueous Nile Blue Sulfate or Neutral Red was added to 30 ml of sea water. Freshly hatched *Artemia* was left in this staining medium for 24 hours. They were then picked in a hand-held micro-pipette and presented within the tentacle range of the colony to be stained: red to one colony and blue to the other colony of a binary combination. Saturation feeding once a day for 6–10 days produced enough color in the growing stolons to determine the origin of solons in an overgrowth tangle and to detect any exchange of material between the colonies. Reciprocal staining procedures were initiated in several incompatible pairs to negate the possibility of chemical involvement of the dye in hyperplastic stolon development. When normal feeding was resumed, the vital dyes faded in about two weeks without apparent harm to either colony.

Controlled breeding was accomplished by isolating sexually mature female colonies at least two days before mating. A slide containing a male colony was introduced into the staining dish, facing the remale colony at a distance of 1–2 cm. Eggs were observed on the bottom of the dish the next morning and elongated planula larvae 12–18 hours later. If larvae were allowed to remain scattered on the bottom of the breeding dish, metamorphosis into small four-tentacled, feeding hydranths was observed in 10–40% of the larva in 7–21 days. Larvae were also picked up in a micro-pipette and introduced onto the clean shell of a hermit crab, resulting in a higher rate of metamorphosis (30–60%). When a new colony was well established, either on glass or shell, one or more feeding hydranths of normal size were transferred to a glass slide, as described earlier, and a stock colony established. The conditions responsible for the induction of sexuality and metamorphosis are highly unpredictable at this time. Most breeding was done at Woods Hole in July and August, but crosses have been made in Indiana in December, and Crowell (1950) and Hauenschild (1954) regularly raised sexually mature colonies in mid-winter at Tübingen, Germany.

RESULTS

Normal growth

Within twenty-four hours after placing a newly isolated hydranth in contact with a slide, stolons grew out along the glass surface, firmly attaching the hydranth to the substratum. Stolons grew in all directions, branching and anastomosing freely within the two dimensions of the slide. They displayed no gravitational tropisms and always maintained contact with the substratum. Other hydranths sprang up along the stolons, increasing the feeding and growth potential of the new colony. The area between the branching and anastomosing stolons was subsequently filled with tissue, histologically described (Berrill, 1953; Bunting, 1894) as an ectodermal mat penetrated by interconnected, endodermally lined gastrovascular channels.

The rate and pattern of stolon growth and mat formation varied among the different strains being raised under identical culture conditions. This fact was also noted by Schijfsma (1939). The same growth patterns appeared in all colonies derived from the same original colony isolated from nature.

Alterations in the culture conditions brought about changes in these developmental patterns. Lack of water movement, reduced aeration and infrequent water changes led to a retardation of stolonic outgrowth, but not to a concomitant reduction in the rate of hydranth production. The result was a smaller colony with a greater density of nutritive zooids. Return of the colony to standard culture conditions produced a renewed stolonic outgrowth similar to that seen in control colonies of the same strain. Cleaning the slide surface and cutting away infected perisarc restored normal fusibility.

Growth on the surface of a gastropod shell was slower than on glass, and mat formation followed more closely behind stolon growth. In shells occupied by a crab, spines were produced after three or four weeks, but on empty shells no spines were produced, although the colony grew well and even reached sexual maturity in some cases.

Overgrowth (hyperplastic stolons)

In order to test compatibility, colonies of two strains were started on a slide, as described earlier. Each developed normally, sending branching, anastomosing stolons in all directions, always maintaining contact with the glass surface, until stolons of one colony made contact with stolons of the other colony. At this point, one of the colonies started to produce abnormal stolons; they rose up off the slide, losing contact with the substratum, and formed hyperplastic, tangled masses. They did not immediately fuse with other stolons of the same colony as they normally would have done when growing flat on the glass surface. During all the tangling, they maintained the general direction of growth toward, and over the other colony. The hyperplastic stolons grew over the stolons, mat and feeding hydranths of the other colony, cutting off its contact with the food supply and eventually causing its death.

Closer observation of the overgrowth phenomenon indicated that physical contact of incompatible strains was essential to the induction of hyperplastic growth, and only those stolons in contact with the overgrown colony were affected. Although material was seen to circulate throughout a colony via the gastrovascular system, hyperplastic stolons were not observed in other areas of the overgrowing colony. Attempts were made to induce the production of stolonic overgrowth throughout a colony by immersing it in a crude brei made from another colony known to induce overgrowth by the contact method, but no positive results were obtained.

Although no abnormal growth patterns were observed in other areas of a colony involved in overgrowth production in the contact stolons, it was noted that normal growth was quantitatively reduced, while the rate of growth of stolons actively involved in overgrowth was increased. The colony as a total unit appeared to be concentrating its corporate nutritive resources in the production of the stolonic tangle at the expense of normal growth in other areas. It was also observed that colonies actively involved in the overgrowth process showed delayed sexual maturation, as compared to control colonies of the same strain.

A tangled mass of stolons could reach a height of 5 mm above the surface of a slide and extend 25 mm across an overgrown colony. When these hyperplastic stolons completely covered the underlying colony and reached the glass surface on the far side, they immediately returned to their normal, two-dimensional, anastomosing growth pattern, regardless of hyperplastic growth still in progress in lateral areas not yet completely covered.

The overgrown colony did not die immediately. First, the feeding hydranths were reabsorbed in the area initially covered by the stolonic tangle. As the overgrowth progressed, more and more nutritive zooids were reabsorbed until all were gone, leaving the overgrown colony with no means of obtaining food. There were, however, large food reserves within the mat, and if the overgrowth was stripped away before too much of these reserves had been utilized, the overgrown colony could again produce feeding hydranths and resume normal growth with no obvious ill effects. If the tangle remained, the tissue of the overgrown mat slowly utilized its food reserves and died within 3–6 weeks. During this time, the stolonic tangle above remained static, while normal growth and hydranth production continued on either side of the tangled mass. Anastomosis of the upper surface stolons of the tangle was observed at about the same time we assume death occurred in the underlying tissue. Observation of cross sections of the mass, using the dissecting microscope, revealed a spongy center of empty perisarc that formerly contained stolons of the overgrowth. The surface was a continuous mat of living ectoderm containing numerous channels lined by endoderm. These channels became clearly defined when vital stain was applied via the food source.

Spontaneously, over the entire irregular surface of the mass, feeding hydranths appeared, with the same density and morphology as those seen in areas of normal growth. If the colony had shown retarded sexual development, gonozooids appeared, evenly distributed on the flat, normal mat and on the surface above the tangle.

No transfer of colored material could be detected between incompatible colonies that had been vitally dyed in contrasting colors, even as the overgrown colony diminished in volume, no absorption of colored material was noted in the overgrowing colony.

Colonies derived from the same source retain their compatibility even after long periods of separation. Fusion was observed between colonies derived from two older colonies of strain #8, Group II, which had overgrown strain #2 and strain #7 respectively, and had been isolated as individual colonies for ten months. Their temporal separation and physiological activity in the overgrowth process had not interfered with or altered their compatibility (fusibility). In similar tests, hydranths from the top of a tangled mass were explanted to a slide between explants of the two strains whose interaction had given rise to the tangled mass of stolons. In all nine cases, the tangle explant colony fused with the colony that had produced the overgrowth and it, in turn, produced hyperplastic stolons when contact was made with the overgrown strain, again indicating no alteration in tissue compability as a result of participation in the overgrowth process.

In rare instances, both colonies produced abnormal stolons upon contact, but one eventually outproduced and overgrew the other. There were situations in which related, but not genetically identical colonies, such as parent and offspring, or two strains with one common parent, contacted each other; each produced a limited number of abnormal stolons, which were quickly replaced by an abnormal, thickened area of mat tissue on both sides of the line of contact. Vital staining showed that there was no fusion or transfer of material between the two colonies, but neither was there any hint of overgrowth by either one or the other.

Hierarchy

In selecting ten colonies at random from nature and setting up all (45) possible binary combinations, it was observed that if colony A overgrew colony B, and if colony B overgrew C, it could be predicted that colony A would overgrow colony C. There was a definite, predictable hierarchy of overgrowth, the strongest overgrower being listed as #1 in the hierarchy. The strain that was overgrown by all the others was designated as tenth in the hierarchy. The other strains all fell in order, depending on their relative frequency as an overgrower.

TABLE I

Results of binary combination—Group I

Strain	Sex	Overgrows	Is overgrown by	Rank in hierarchy
2	♂	1 3 4 5 6 8 9 10	$\boxed{7}$	1
5	♂	3 4 6 7 8 9 10	2 ①	2
3	♂	1 4 6 7 8 9 10	2 5	3
8	♀	1 4 6 7 9 10	2 3 5	4
7	♀	1 $\boxed{2}$ 4 6 9 10	3 5 8	5
1	♂	4 ⑤ 6 9 10	2 3 7 8	6
9	♀	4 6 10	1 2 3 5 7 8	7
4	♂	6 10	1 2 3 5 7 8 9	8
10	♀	6	1 2 3 4 5 7 8 9	9
6	♀		1 2 3 4 5 7 8 9 10	10

☐ + ○ are not in expected positions.

In the first set of data presented in Table I, there are two discrepancies in the hierarchy. Strain #7 was scored as overgrowing #2, and #1 was scored as overgrowing #5, which is contrary to expectations based on data derived from the other 43 binary combinations in this group. Neither of these results could be checked due to an accidental loss of all Group I strains. There was one apparent discrepancy in the predicted results of Group II. In the first trial, #7 appeared to overgrow #8, but subsequent tests of the pair, both on slides and crab shells, accompanied by vital staining, proved #8 to be the overgrower as would be predicted from other tests, leading to the possibility that there may have been a labeling reversal in the initial trial.

Although two strains proved incompatible upon stolonic contact, this did not affect sexual interaction. It was, therefore, possible to produce second generation strains. There were five matings in Group I and three in Group II.

The larvae were allowed to metamorphose on the bottom of the breeding dish. As growth continued it was observed that stolonic compatibility and incompatibility existed between siblings and that either fusion or overgrowth occurred at the junction of any two colonies. By transferring colonies to slides and setting up from nine to twenty-four possible combinations per mating, it was determined that only two classes of offspring existed: the overgrowers (*i.e.,* 7–4(0)) and the overgrown (7–4(x)). All those that overgrew their siblings were compatible, and all those that were being overgrown were fusible with each other. Because of the large numbers of larvae and limited space, no accurate determination of the percentage of offspring in each class was made.

TABLE II

Results of binary combinations—Group II

Strain	Sex	Overgrows	Is overgrown by	Rank in hierarchys
10	♀	1 2 3 4 5 6 7 8 9	—	1
8	♀	1 2 3 4 5 6 7 9	10	2
9	♀	1 2 3 4 5 6 7	8 10	3
4	♂	1 2 3 5 6 7	8 9 10	4
1	♂	2 3 5 6 7	4 8 9 10	5
7	♀	2 3 5 6	1 4 8 9 10	6
6	♀	2 3 5	1 4 7 8 9 10	7
5	♂	2 3	1 4 6 7 8 9 10	8
3	♂	2	1 4 5 6 7 8 9 10	9
2	♂	—	1 3 4 5 6 7 8 9 10	10

DISCUSSION

The initial work on *Hydractinia* was basically descriptive of normal development (Bunting, 1894; Teissier, 1929; Teissier and Teissier, 1927; Schijfsma, 1935, 1939; Berrill, 1953). Teissier (1929) and Schijfsma (1939) noted that there was fusion into a single colony when stolons derived from different planula larvae made contact, but an anomaly was noted by Schijfsma in 1939 (page 101): "It looks as if the growing borders of two colonies, in striking together and checking each others progress, are stimulated to very active growth and ramifications; resulting in the formation of a dense fringe of intertwined stolons."

Schijfsma vaguely speculated about a "timing factor'" but noted that this "fringe" did not appear when a colony met itself on the other side of a shell. This indicated that the "fringe" was not a normal marginal phenomenon. Toth (1967) enlarged upon this suggestion, calling it "temporal specificity" (page 131), and claiming that compatibility, even of clonal colonies was variable with time. He stated that all colonies were compatible early in life, but became increasingly selective; eventually colonies of the same strain could not fuse. In this study, however, incompatibility was demonstrated between newly metamorphosed (2–3 day) colonies by the production of hyperplastic stolons which overgrew sibling colonies; while continued compatibility was demonstrated by the fusion of explant colonies derived from two colonies of the same strain that had been established as individual colonies ten months earlier. Both colonies had undergone the physiological stresses of overgrowth production and still retained their compatibility. Colonies derived from hydranths on the surface of the tangle fused with clonal colonies of the strain that had originally produced the tangle, indicating that even stolons that initially appeared unable to fuse during the overgrowth process retained their histo-compatibility when they returned to normal colonial metabolism.

Another point of variance in Toth's paper (1967) was his report that colonies on glass slides usually reached a maximum diameter of 5–10 mm before the "endogenous limit of closed periderm is attained" (page 131). He does, however, mention later that no such limit is seen in nature. No such limits were seen in the present study. A "dirty" slide whose surface is covered by a layer of bacteria, algae and/or protozoa will inhibit or halt free stolon growth. The vulnerable stolon, with its high surface-to-cell-mass ratio, is poisoned or damaged by these other organisms faster than it can regenerate new tissue, and eventually new stolon growth stops. The mat may slowly expand for a while longer under these conditions, but this too eventually ceases. Toth's (1967) description of a "limiting periderm" and reduced stolon growth can be explained as an artifact of substandard culture methods. His report of incompatibility (lack of fusion) between colonies of the same strain may be due to a build-up of necrotic tissue or bacteria-encrusted perisarc at the contiguous margins of the two colonies, preventing perisarc dissolution by ectodermal enzymes or preventing cell-to-cell contact and tissue fusion.

Toth (1966, 1967) reported free stolon growth in 10% of his strains. Both Hauenschild and Kanellis (1953) and Toth (1967) suggest this may be due to poor nutrition. The current study of well fed and well aerated stocks produced an open stolon pattern in 80% of the strains tested, with a varying stolon/mat ratio characteristic of each strain and reliably reproduced by all colonies derived from that strain. Reduced oxygen supplies in the medium retarded stolon growth. The mat continued to grow as a slowly expanding circle from the point of implantation. A return to more advantageous culture conditions brought a renewed outgrowth of freely anastomosing stolons.

Crowell (1950), Hauenschild (1954), and Toth (1967) discuss lack of fusion between strains and regard this as incompatibility, but none of these investigators records or discusses the induction of hyperplastic stolons. Müller (1964), however, does report the formation of stolonic "knots" to which both colonies contributed stolons.

The role that particular strain plays in relation to any other strain, either as overgrower or overgrown colony, is not a chance occurrence. Among the strains tested, a very definite hierarchy emerged in both sets of experiments.

There appeared to be a correlation between growth potentials, as related to colony morphology, and the position of a strain in the hierarchy. A fast-growing, highly stolonic strain is likely to rank higher on the scale of overgrowth potential than a slower-growing, short-stolon, large-mat former; the correlation is not absolute, however, and rank by growth rate becomes difficult to determine among strains of similar developmental morphology.

Vital staining experiments showed that in some cases both colonies initially produced abnormal stolons. Müller (1964) reported the participation of both colonies in the formation of a stolonic "knot." In almost all cases, one strain was superior in hyperplastic stolon production and the other began a regression that ended in death. Müller suggests that this regression is caused by a toxin produced by the overgrowing colony. Stripping away the overgrowth leads to rapid (2–4 days) and full recovery, suggesting that the regression is the result of mechanical stresses applied by the hyperplastic stolons. When 80% of the colony being overgrown is covered, the other 20% spontaneously withdraws the feeding hydranths in the uncovered area. It is suggested that this is the result of a general physiological regression of the entire colony caused by an unfavorable balance between metabolic requirements and nutritional acquisition rather than a reaction to a specific toxin.

The term "hierarchy" suggests the work of Steinberg (1963) and his hierarchy of embryonic tissue associations and segregations in tissue culture. His results indicated a predictable position in a cellular reaggregate. Pre-cartilage had the highest probability for interior position, liver the highest probability for the outside, with heart-cells variable, based on the particular binary combination. Steinberg repeated these experiments with several embryonic tissues establishing a hierarchy of potential position at the center of the mass. The explanation offered by Steinberg involved an "energy of adhesion" between cells, so that if two cells of type A displayed a significantly higher attraction for each other than did those of type B or an A cell for a B cell type, the A type cells would tend to aggregate together with as much mutual surface contact as possible, thereby excluding cells of the B type from their midst and forcing them toward the periphery of the reaggregate cell mass.

"Energy of adhesion" is a cell surface phenomenon in which cells seek the "lowest energy state" or most stable adhesive condition possible. The adhesive mechanism could, in principle, be a quantitative one based on the number of adhesive sites available or a qualitative one based on the specificity of the various sites.

Applying these hypotheses to the hierarchy in *Hydractinia*, two possible mechanisms may be proposed, both involving a surface-bound molecule produced by the ectoderm. The first hypothesis involves a quantitative variation in this substance; the second suggests a qualitative difference. When stolons of the same strain meet, the quantity and/or quality of the molecule is identical and fusion results. If, on the other hand, there is a significant difference in either quantity or quality of the substance, the stolons recognize this difference and react by the production of hyperplastic stolons by one or both colonies.

Looking first at the qualitative hypothesis, we can postulate a mechanism similar to serotypes found in *Paramecium*. (Sonneborn, 1948). Incompatible strains would produce strain-specific proteins which could induce hyperplasia in other strains. The intensity of the reaction could be due to the degree of difference in the surface molecule. It was noted that the intensity of incompatibility, as indicated by the speed and quantity of induced hyperplastic growth, varied considerably among the strains tested. Certain slow-growing strains, such as 4, 6 and 10, Group I and strains 2 and 3, Group II, induced a much weaker reaction from the #1 strain in their respective hierarchies than did other overgrown strains higher up in rank.

The maximum degree of difference that will trigger the reaction is limited, however, as evidenced by the fact that contact with other hydroids (*i.e., Campanularia, Bougainvillia* and *Podocoryne*) failed to elicit a response. This indicated that the overgrowth reaction was not a simple antigen-antibody-like response to a foreign protein, but rather a highly specific, intra-species selective mechanism that plays a role in genetic distribution within the *Hydractinia* population.

Considering the quantitative difference hypothesis, the colony with the higher concentration of the particular molecule in any pair might be the inducer. This would explain a strain's shift from overgrower to one which is overgrown as a shift in the relative amount of this surface molecule, when compared to the quantity of this substance in the other strain of any particular combination. This would be similar to Steinberg's hypothesis of differential adhesion based on a quantitative difference in available binding sites. The hierarchy then would be a quantitative ranking of the presence (or absence) of this inducer molecule.

Preliminary experiments (Ivker, 1967) with reaggregation of dissociated, stained endoderm cells do not demonstrate histo-incompatibility between strains on the cellular level.

Braverman, M. (Allegheny General Hospital, Pittsburgh, Pa.) has photographic and histologic evidence that even in normal stolon fusion, in the related encrusting species *Podocoryne,* the advancing stolon tip produces a substance that causes an increase in the size of the epidermal cells that the tip is about to contact. An increase in epidermal cell size is also seen in the overgrowth stolons. Müller (1964) mentions a hyperplasia of epidermal cells in both the stolons and the mat in the area of contact between incompatible strains.

It is suggested that each strain produces a substance (in greater quantity at the growing tip) which has a hyperplastic effect on epidermal cells. In the contact of stolons of the same colony, or colonies of the same strain, the stolon tip is thought to produce an enzyme which dissolves the perisarc in a small area and facilitates cell-to-cell contact and fusion of the gastrovascular cavities. When strains are incompatible, the surface substance on the growing stolon again induces a reaction in the epidermal cells in the area of contact. These cells produce more of their own surface substance which in turn induces hyperplastic growth in the oncoming stolons. This accelerated growth rate may prevent the accumulation of sufficient enzyme at the stolon tip, thereby preventing the fusion of actively growing hyperplastic stolons with each other. Müller (1964) proposes a similar mechanism (page 241) when he ascribes "wild" stolon growth to a stimulation of dormant developmental potential in one strain by a foreign (incompatible) strain.

Attempts to characterize the inducing substance have given rise to several hypotheses. It is either bound to the ectodermal surface or it slowly diffuses through the perisarc from the ectoderm. The failure of an incompatible strain to "condition" the medium in which it was grown, and the failure of a crude brei of incompatible colony to induce hyperplastic stolon development may be evidence of the small quantities produced by any given colony and/or the failure of the material to reach a concentration above the threshold required for hyperplastic induction.

As was noted earlier, stolonic contact between incompatible strains was essential for the induction of hyperplastic stolons. Only growing stolons were affected (*i.e.,* those laid down before contact was made remained unaffected). Nothing was carried via the gastrovascular system to induce abnormal stolon development in any area of the colony not in direct contact with the overgrown colony, leading to the hypothesis that the overgrown colony acts as an inducer. The inducing agent is either surface bound or a large molecule that cannot easily diffuse through tissue or water in sufficient concentration to induce overgrowth in any but contiguous stolons. There must be continued production of the substances, since the stolonic mass bult up, but remained as a deep tangle of stolons as long as there was living inducer colony below it. Stolons that reached the far side of the overgrown colony and made contact with clean glass, resumed their normal growth pattern and regular hydranth production. Although stolons in the tangle acted as connectives between colony mat on either side of the overgrown colony, no inductive material was transported in either direction to affect hydranth production. Hyperplastic growth was localized to that area in direct contact with the incompatible colony. The only area lacking feeding hydranths and not displaying stolon anastomosis and mat formation was the tangle mass itself and this situation was temporary. The eventual stolonic fusion, mat formation and appearance of nutritive polyps was believed to coincide with the death of the overgrown colony and the cessation of its production of inducer. It is suggested that the inducer substance promotes increased growth (*i.e.*, hyperplastic stolons that do not anastomose) yet it inhibits differentiation of specialized tissue areas like feeding or reproductive zooids. Perhaps the increased growth rate prevents the concentration of material required for hydranth formation.

Although there was no gross morphological change observed in the outlying parts of the overgrowing colony, there was, nevertheless, an effect felt throughout the colony. The hyperplastic mass resulted from an accelerated deposition of material in one area, which could only be made at the expense of growth in other areas. The stolonic tangle increased the mass of the colony, but the nutritive capacity of the colony did not undergo a concomitant increase, due to the absence of feeding hydranths in the tangle area. There was, therefore, a definite decrease in peripheral stolon growth and mat production as compared to control colonies. There was not total cessation of normal growth patterns, but a noticeable retardation as the colony concentrated its productive energy and nutritional resource in the overgrowth mass.

Although the induction of sexuality is far from understood, optimal nutrional conditions are a prerequisite to the process. Sudden starvation caused the transformation of gonozooids into nutritive polyps. The reverse was not true. Gonozoiids arise *de novo* from the mat tissue and were not derived from pre-existing nutritive structures. It is postulated that the accelerated proliferation of stolon tissue that constituted the overgrowth mass depleted the nutritional reserves ncessary for gonozooid production, thereby retarding sexual differentiation.

Sex does not appear to be related to compatibility. Colonies initially determined to be 7–5(X), Group I immediately after metamorphosis, but raised on separate slides, subsequently turned out to be of opposite sex. Fusion of these colonies after initial growth had established the individuality of each colony could produce sexual chimeras in the fusion zone, as reported by Müller and Hauenschild. Müller (1964) reported having to try many binary combinations (which produced "knots") before he found two strains that were even partially compatible. In this case, he reported fusion of the endodermal gastrovascular cavities, while the mat ectoderm of the female colony formed large masses that appeared to invade the male colony. The masses eventually withdrew. The separation of apparently fused, partially compatible colonies was observed in this study, especially in times of physiological depression. In trying to localize the source of incompatibility, it was found that empty perisarc did not elicit a reaction, indicating that the inductive substance was not an integral part of the molecular structure of the perisarc.

The variability of morphology seen between various strains, the consistency of morphology within a strain and the clear-cut orderliness of the hierarchy indicate a genetic control mechanism. Hauenschild initially proposed a single locus, six-allele system to explain the results of his histo-incompatibility studies, but ultimately abandoned the hypothesis when it proved inadequate to deal with the complexity of accumulated data.

Looking to other biological systems for a clue, the colonial tunicate *Botrylus schlosseri* presents a seemingly similar example of histo-incompatibility in which the vascular systems of incompatible strains fail to fuse, but no hyperplastic growth is observed. Karakashian and Milkman (1967), Milkman (1967) postulate a multi-allelic system, but assign no definite number of alleles.

At the present time, there is no definite evidence to support a multi-allelic versus a multi-genic hypothesis, or to rule out a complex combination of the two. With the demonstrated feasibility of controlled laboratory mating and the use of morphologically unique strains, it is hoped that some insights will soon be gained into the mechanisms of the genetic transmission of incompatibility.

The ability to produce hyperplastic stolons and a high place on the hierarchy appears to have a selective value in nature. It is hoped that morphologic markers can soon be found that can help trace the ecological distribution of the genetic factors responsible for the overgrowth phenomenon, and that the inductive mechanism can be more firmly established.

SUMMARY

1. *Hydractinia* represents a simple system in which to study induction, cellular-recognition mechanisms, and hyperplastic growth.

2. Among various strains isolated from nature, there is a tissue incompatibility upon contact which results in the production of hyperplastic stolons (overgrowth) in one or both colonies of any binary combination of strains. The induction of hyperplasia probably involves surface-bound molecules produced by the ectoderm.

3. A hierarchy of hyperplastic potential was established in two groups of ten strains each. A correlation between colonial morphology and rank in the hierarchy was noted.

4. Consistency of morphology and intermediate forms of incompatibility between related strains (*i.e.*, parent-offspring, half-sibs) suggests genetic control of histo-incompatibility and hyperplastic growth.

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SPATIAL DISTRIBUTION OF *NUCULA PROXIMA* SAY (PROTOBRANCHIA): AN EXPERIMENTAL APPROACH

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The spatial patterns of populations are influenced by many of the important physical and biological parameters that control the abundance and behavior of individuals of the population. For instance, strong environmental heterogeneity tends to cause a clumped distribution. This is especially true of rare species, since their preferred habitat is likely to occur patchily in the overall biome (Hairston, 1959). Strong negative interactions between individuals due to direct interference or territoriality tend to cause a uniform distribution (Connell, 1963; Holme, 1950; Johnson, 1959; and others). Random distributions may result from random settling of larvae from the water column (Connell, 1955), an abundance of resources (Hairston, 1959), random movements of individuals of the population, or a random distribution of resources. Because of the patchiness of most natural environments, most populations are aggregated in their spatial pattern. The classic study of Holme (1950) showed territoriality in a deposit-feeding Tellinacean bivalve.

Nucula proxima Say (Protobranchia), an infaunal deposit-feeding bivalve was studied in both the laboratory and the field. This species was studied in the laboratory by the use of a technique employing x-radiography. By x-raying trays of sediment containing individuals of this species all individuals can be located exactly. Also, substratum heterogeneity, water characteristics, size of individuals and population density can be manipulated. All of these factors make laboratory studies of spatial patterns, where possible, highly desirable as complementary data to field studies.

METHODS AND MATERIALS

Approximately 5000 individuals of *Nucula proxima* were collected with their native substratum from a depth of 20 m in Long Island Sound, off Milford, Connecticut. Individuals of the populations collected were of a size and morphology confined to very high silt-clay sediments in Long Island Sound and Buzzards Bay. This species is probably different from those forms living in sandy sediments of Buzzards Bay, Massachusetts (Hampson, verbal communication). Individuals were acclimated for two weeks or more in Instant Ocean Aquaria at 15° C, using Long Island Sound sea water (salinity $\sim 29‰$). This experimental substratum had a silt-clay content of over 90%, typical of natural *Nucula* substrata in Long Island Sound and Buzzards Bay, Massachusetts.

For each experiment, square trays 7.5, 10 or 15 cm on a side (0.5 cm deep) were filled with homogenized sediment. *Nucula* was then placed on the surface of the mud. Initial movements and subsequent vertical and lateral burrowing quickly eliminated the initial spatial pattern (which was usually aggregated). The range of densities approximated 5×10^3 per square meter; the mean density for Station R, Buzzards Bay, Massachusetts (Sanders, 1960).

Trays were kept in 25 gallon recirculating Instant Ocean Culture Systems. Water movements were minimized with plexiglas partitions in the aquarium. After an elapsed time of 10–89 days a tray was removed and placed on a piece of Kodak Industrial x-ray film, and x-rayed vertically (in air) with a medical x-ray unit. Because the bivalve shell is relatively opaque to x-rays, the positions of individuals are clearly recorded on film (Fig. 1). This technique was developed by D. C. Rhoads (see Rhoads and Young, 1970; Stanley, 1970) for the *in situ* study of infaunal species.

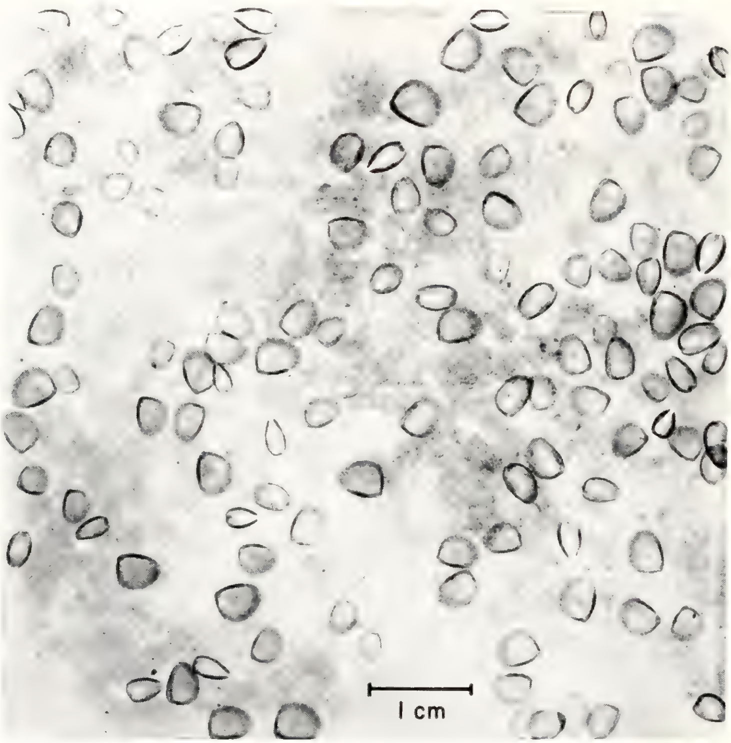

FIGURE 1. X-radiograph of a dispersion experiment.

The x-radiograph of each tray, regardless of size, was divided into 64 quadrats and the number of animals per quadrat were counted. The fit of the observed patterns with a Poisson distribution was tested by using the Chi-Square goodness-of-fit test.

In addition, two distance measures were used to test for departures from random patterns. One was the distance from an animal to its nearest neighbor (Clark and Evans, 1954). The second was the distance from a randomly located point to the nearest animal (Morisita, 1954; Cottam and Curtis, 1956). If the population is randomly arranged, then the mean distance of this parameter should be the same as for the distance from an individual to its nearest neighbor (Morisita, 1954). A test of significance suggested by Thompson (1956) was employed in both cases.

For a field comparison, *Nucula proxima* was collected at "Station R," Buzzards Bay, Massachusetts (Sanders, 1960). A multiple-tube sampler, similar to that of Buzas (1968), was constructed by cementing plexiglass cylinders of circular cross-section (diameter = 4.7 cm) so that the sampler consisted of 36 contiguous tubes, arranged in a square pattern (Fig. 2). To collect the sample, the sampler was inserted in the bottom. It was then capped with a cover made of foam rubber backed by plexiglass, and then pulled from the bottom. The suction created by the cap prevented the sediment in the tubes from dislodging.

The samples were collected on August 9 and 19, 1968. The first set was sieved through a 0.71 mm sieve, while the second was sieved through a set of four sieves (2.00, 1.41, 0.70, and 0.42 mm) in order to investigate the differences in spatial distribution between juveniles and adults (Buzas, 1968; Jackson, 1968). In both samples some of the individual core samples were lost due to the difficulty of getting 36 cores at once.

RESULTS

The spatial patterns and experimental condition of the 13 analyses performed are shown in Table I. These experiments were done under different elapsed times. All x-rays but one show that the spatial pattern of *Nucula proxima* is random, with no territoriality or gregariousness detected. The exception shows an aggregated pattern. Upon inspection of the x-rays, it was obvious that there was significant movement of animals to one side or corner of the tray, rather than aggregation into small clusters. The aggregation is on a very large scale; equal in magnitude to the size of the tray. Therefore, the aggregated pattern is probably due to failure to homogenize the experimental environment, rather than gregariousness in *Nucula proxima*.

One experiment was tested for the effect of change in quadrat area. The tray was divided into 32, 64, 128, and 256 quadrats. In all cases, when compared to a Poisson distribution, the distribution of abundances did not differ significantly from randon (Table II).

Using a method proposed by Clark and Evans (1954), the distance of each individual to its nearest neighbor was measured by determining coordinates for the animals' locations, and measuring the distance from a point to its nearest neighbor. This was done by use of a computer program (on file) which selected the minimum distance of a given point to all other points in the experiment. In experiment number 10, the mean distance was found to be significantly greater ($P < 0.05$) than would be expected in a random distribution (Thompson, 1956). In other words this test indicated uniformity in the populations' spatial pattern. However, the pattern of experiment number 11 (same tray, 47 days later) did not differ significantly from random ($P < 0.05$).

In experiment number 10, and in cases where nearest neighbor analyses of randomly constructed patterns proved to be uniform, the frequency distribution of

TABLE I

Conditions of the experiments and resultant spatial patterns determined by comparison of 64 quadrat results with Poisson distribution

Expt. #	Elapsed time (days)	# of animals	Tray side (cm)	Area (m^2)	#/m^2	Mean temp. C	Computed χ^2	χ^2 ($P = 0.95$)	Spatial pattern R = random A = aggregated U = uniform
1	79	87	15.0	0.0225	3.9×10^3	10.0	0.0385	7.815	R
2	32	90	15.0	0.0225	3.9×10^3	9.9	0.5485	7.815	R
3	10	92	15.0	0.0225	4.0×10^3	6.2	1.2993	7.815	R
4	29	96	15.0	0.0225	4.3×10^3	19.3	0.2499	7.815	R
5	29	97	15.0	0.0225	4.3×10^3	19.3	3.5965	7.815	R
6	89	92	10.0	0.0100	9.2×10^3	9.5	0.8137	7.815	R
7	10	225	15.0	0.0225	1.0×10^4	6.2	3.2413	11.143	R
8	35	117	10.0	0.0100	1.2×10^4	19.8	1.6825	11.143	R
9	35	124	10.0	0.0100	1.2×10^4	19.8	5.4929	7.815	R
10	32	298	15.0	0.0225	1.3×10^4	9.9	5.2118	12.592	R
11	79	298	15.0	0.0225	1.3×10^4	10.0	3.6320	12.592	R
12	89	222	10.0	0.0100	2.2×10^4	9.5	1.9495	11.071	R
13	10	132	7.5	0.0563	4.1×10^4	6.2	12.5868	11.143	A

nearest neighbor distances was right-skewed. However, in the patterns not differing significantly from the Clark and Evans null hypothesis of randomness, this frequency distribution was closer to normal. The statistical test proposed by Clark and Evans (1954) requires such a normal frequency distribution.

A similar test involves locating points randomly within the area in question, and measuring the distance from each point to the nearest animal (Morisita, 1954; Cottam and Curtis, 1956). Using this test on experiment 11, the mean distance was not found to differ from that mean distance expected in a random distribution ($P < 0.05$).

The point-centered quarter method was also employed on this experiment. This consists of dividing an area around randomly selected points into quadrats. The distance from the point to the closest animal in each quadrat is measured. This method showed the spatial pattern of this experiment to not differ significantly from random ($P < 0.05$).

Spatial patterns, particularly uniform ones, are strongly affected by the variance in body size of the population. Pielou (1960) showed that organisms that are "territorial" will yield an aggregated distribution if the range in size is so great

TABLE II

Effect of differing quadrat areas on spatial distribution in experiment #11/ computed χ^2 is based on comparison with the Poisson distribution

# of Quadrats	Computed χ^2	χ^2, $p = 0.95$	Spatial pattern
32	0.1271	7.815	R
64	3.6320	12.592	R
128	1.6107	11.071	R
256	3.0392	9.488	R

as to produce a corresponding range in distance to nearest neighbors. This factor is inferred to operate if there is a correlation between nearest-neighbor distances and the sum of their sizes (Connell, 1963). Two experiments were tested for this effect. In both analyses (analysis 1: $r = -0.154$, d.f. $= 33$, F $= 0.798$; analysis 2: $r = 0.054$, d.f. $= 33$, F $= 0.095$) no significant correlation was found.

In conclusion, it is judged that *Nucula proxima* maintains a random distribution in a homogeneous experimental environment.

The two field samples from station R show that the spatial pattern of *Nucula proxima* in its natural habitat is also random. One sample was sieved core by core into 4 size fractions (2.00 mm, 1.4 mm, 0.77 mm, and 0.42 mm) and these groups were evaluated individually. The spatial pattern for all size fractions combined was evaluated for both sample sets. In both sample sets only 29 cores were recovered successfully. The results of the analyses are shown in Table III. In all cases but one the spatial pattern is random. The one exception, size fraction c, is aggregated. There are slight tendencies in all of the field samples towards aggregation; being probably an indication of the heterogeneity of the environment. It must be remembered that the total area sampled by each core set is equal to about twice the area of the largest experimental tray. Thus the chances for environmental heterogeneity are greatly increased, especially since the environmental area is by no means approximately homogeneous; as in the experimental trays. These results clearly show the need for contrasting lab and field studies of spatial

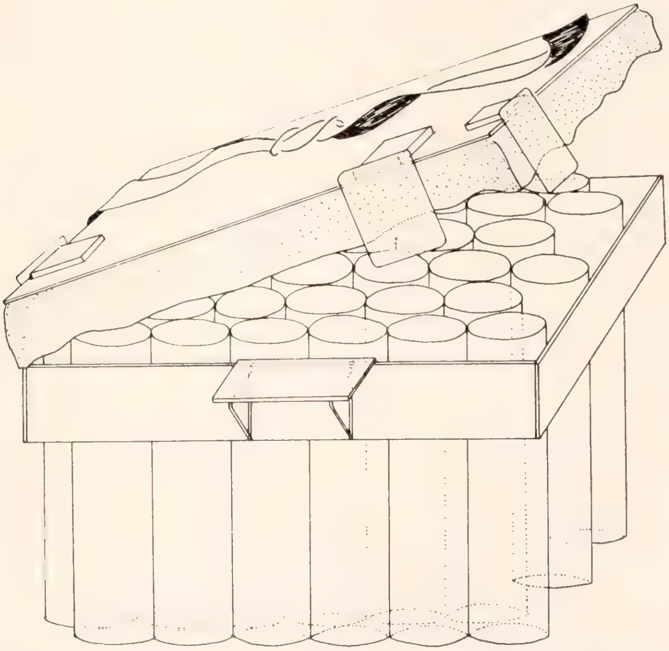

FIGURE 2. Multiple-coring device used in collection of Station "R" samples. Diameter of each core is 4.7 cm.

patterns. With laboratory investigation, one can learn those interactions which are due solely to positive or negative interactions between individuals; and not to environmental heterogeneity.

DISCUSSION

Nucula proxima is a deposit-feeding bivalve, feeding by means of a tentacle-like palp proboscide which conveys detritus to the mouth by means of a ciliated groove. It feeds within the substratum, rather than grazing on the surface for particles delivered by the overlying water. The abundance of *Nucula proxima* in Buzzards Bay and Long Island Sound correlates with parameters related to the availability of food (Sanders, 1956, 1958). Therefore, populations of *Nucula* are probably food limited, and at the environment's carrying capacity. This food limitation might produce the expectation that *N. proxima* would be territorial (*i.e.* maintain a uniform dispersion pattern) in order to ensure a predictably available food source. The following arguments, however, indicate that there should be no selection for territoriality in fairly dense populations of this species.

TABLE III

Analysis of polycorer data, comparing core abundance frequencies with those expected in a Poisson distribution; using the Chi Square goodness-of-fit test. R1 taken August 19, 1968; R2 taken August 8, 1968. A, B, C, and D refer to 2.0, 1.4, 0.7, and 0.4 mm fractions, respectively. Total populations are "t" samples

Samples	R1A	R1B	R1C	R1D	R1t	R2t
Calculated χ^2	2.37	1.34	6.27	3.61	2.64	0.99
$\chi^2 0.95$	3.84	5.99	5.99	5.99	5.99	3.84
Spatial pattern	R	R	A	R	R	R

(1) There is no known available means of territorial defense for *Nucula* to employ. In addition there is no readily available mechanism to allow this species to sense and interact with other individuals of the population. Unlike species of *Tellina* (Bivalvia: Tellinacea), which have long, mobile siphons, *Nucula* communicates with the outside medium only with its relatively short palp proboscides and has no siphons. In addition, *Nucula* maintains no permanent burrow.

(2) Furthermore, consider a fairly dense population of deposit-feeders who move about constantly. When an individual leaves his former area of foraging, he has two choices. He can either return to his own area, or move into the area of another individual. If the population is fairly dense, it is probable that the area of the second individual has been just as completely (or incompletely) exploited as that of the first. Therefore, there is no relative advantage or disadvantage to turning about and feeding upon a home territory or continuing into another individual's feeding area. This is especially true as *Nucula* can only feed on a relatively small area at a time, and has to keep burrowing around. This is in contrast to deposit-feeding Tellinacean bivalves, who in stationary life position can feed on a relatively large area with their long, mobile siphons. In this case it is energetically more economical to maintain a territory and stay in one place

(since the feeding area is large) than to move about. This is especially true for those forms whose food source is delivered by currents to the bottom. These above arguments probably explain why *Tellina tenuis* and *T. agilis* seem to be territorial and maintain uniform distributions (Holme, 1950; William Gilbert, verbal communication); whereas *Nucula proxima's* spatial pattern is apparently random.

(3) A third argument leading to the expectation of randomness in Nucula spatial patterns involves the stability and water content of the sediment. The reworking activities and fecal pellet formation of *Nucula proxima* increases the water content of the sediment and produces an unstable, fluid medium (see Rhoads and Young, 1970; Levinton, 1971). In the presence of weak bottom currents the fluid substrate is easily suspended, making the stability of *Nucula's* living position very unpredictable. Under these conditions, it is unlikely that selection for behavior relating to territoriality would be at all important.

There is no difference in the spatial patterns of small *vs.* large individuals in the field samples. Others have found that juveniles are often aggregated, whereas adults are random or uniform. Jackson (1968) and Buzas (1968) ascribed this to parental release of offspring, leading to parent-offspring aggregations. Green and Hobson (1968) claim that this initial aggregation, in the case of *Gemma gemma* (Bivalvia, studied by Jackson, 1968) would be soon eliminated by tidal currents, or would be altered by substrate heterogeneity. Gilbert (1968) found these same dispersion phenomena in natural populations of the Dward Tellin Clam, *Tellina agilis.* He demonstrated that the settling velocity, and subsequent aggregation, of juveniles was identical to the settling velocity of sand 0.2 mm in diameter. Sediment of this mean diameter was the substratum of maximum abundance of *Tellina* juveniles. Studies of juvenile *vs.* adult spatial patterns are clearly important sources of data relating to the change of selective forces with increasing age.

Connell (1963) examined and compared the spatial patterns of several marine benthic invertebrate populations, of widely differing ecological types. He noted that *Uca pugilator*, an epifaunal deposit-feeding fiddler crab, and *Erichthonius braziliensis*, an epifaunal grazing amphipod, both maintain uniform spatial patterns. Individuals of both species live in permanent dwellings and the need for the defense of territory is obvious. As Connell (1963) also argues, the establishment of territories allows all of the energy of the individual to be channelled into growth and reproduction. *Nucula proxima,* and other mobile infaunal deposit-feeders, do not fit into this category since they do not maintain permanent burrows. In addition, their mode of feeding is different from *Tellina,* which Connell also lists as having a uniform pattern.

In infaunal suspension feeding populations environmental heterogeneity seems to be very important in bringing about clumped patterns (Connell, 1963). The factors which determine the abundance of suspension-feeders are those which often are not systematically distributed in space, or predictable in time. This tends to lead to aggregated distributions. In contrast, deposit-feeders' spatial patterns are strongly determined by their feeding and burrowing behavior. Deposit-feeders tend to condition their substratum, and control its structure (Rhoads and Young, 1970).

The above makes it clear that it is difficult to make comprehensive statements about the spatial distribution of organisms. It is apparent that spatial patterns are controlled by the interaction of environmental heterogeneity, trophic group, mode of reproduction and mobility. However, spatial patterns are invaluable in the investigation of specific problems relating to behavior, environmental heterogeneity and the environmental components of selection.

I am grateful to D. C. Rhoads for provision of equipment, much helpful discussion and continued encouragement. Conversations with W. H. Gilbert were important in the development of this study. Roger Green read the manuscript. I thank J. B. Howard and D. K. Young for collecting samples at Station "R." Finally, I thank H. L. Sanders (Woods Hole Oceanogr. Inst.), whose provision of lab space at Woods Hole made the initiation of this study possible. This study was supported by a Penrose Bequest (Geol. Soc. America) and by a Sigma Xi grant-in-aid.

SUMMARY

The spatial patterns of populations of *Nucula proxima* were studied in the laboratory using x-radiography, and in the field using a multiple-coloring device.

Trays with sediment and varying population densities of *N. proxima* were x-rayed under different temperature and elapsed time conditions. Almost all experiments were shown to exhibit random patterns. The two exceptions were aggregated distributions which were probably due to inhomogeneities in the experimental environment.

The field samples showed *Nucula proxima* to be randomly distributed, with a tendency towards aggregation in some cases. Juveniles were distributed essentially the same as the adults.

It is argued that the lack of defense mechanisms, the instability of the substrate, the small "reach" of the feeding organ, and the lack of advantage of territoriality to a mobile deposit-feeder all contribute to the observed random patterns of *Nucula proxima*.

It is concluded that x-ray studies of infaunal invertebrates done in concert with field studies is an excellent means of distinguishing the factor of environmental heterogeneity from negative or positive interactions between individuals caused by territoriality or gregariousness.

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CHEMORECEPTION IN THE MIGRATORY SEA TURTLE, *CHELONIA MYDAS*

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It has long been suspected on the basis of neuroanatomical (Papez, 1961), neurophysiological (Tucker, 1963; Tucker and Shibuya, 1965) and behavioral evidence (Boycott and Guillery, 1962), that fresh water and land turtles have a sense of smell. Nothing is known of chemoreception in sea turtles, although loggerhead turtles (*Caretta caretta*) have been observed underwater with their nostrils open and the floor of the mouth moving up and down, possibly engaged in chemical sampling (Walker, 1959).

The green turtle (*Chelonia mydas*), whose life cycle has been studied intensively, is known to migrate long distances through the open sea. Tagging studies have demonstrated that populations of Atlantic green turtles usually leave their year-round feeding grounds to mate and breed on beaches that are hundreds of miles away (Carr, 1967). For example, the population that nests on Ascension Island feeds near the coast of Brazil, a distance of 1400 miles. Their method of navigation is unknown. Orientation by visual cues alone seems unlikely, moreover these turtles have been shown to be myopic when their eyes are out of water (Ehrenfeld and Koch, 1967). It has been suggested recently that the detection of chemicals entering the South Equatorial Current from Ascension Island might aid in the navigation of the Brazilian migrants (Koch, Carr and Ehrenfeld, 1969). Carr (1972) has called attention to evidence that olfactory cues might also be available to migrants to a mainland nesting shore.

In this study we used operant conditioning techniques to examine the ability of the green turtle to detect various chemical substances dissolved in water. In addition we tested a method of reversibly interrupting olfaction for a period of days by treating the olfactory epithelium with a 0.35 M solution of $ZnSO_4$.

MATERIALS AND METHODS

The experimental subjects were four immature Caribbean green turtles. At the start of the experiment they were 6 months old and weighed 300 to 450 g. They lived in recirculating artificial sea water and were tested in fresh running water (green turtles are osmotically highly adaptable). The turtles had been hatched and reared in the laboratory from eggs obtained in Costa Rica, and were previously untested. They were kept on a 23-hr food deprivation schedule during the experiments.

The apparatus used for training and testing is diagrammed in Figure 1. The experimental chamber was a tank 30 cm wide, 45 cm long and 30 cm deep containing water at a depth of 7 cm (8 l) which flowed continuously through the

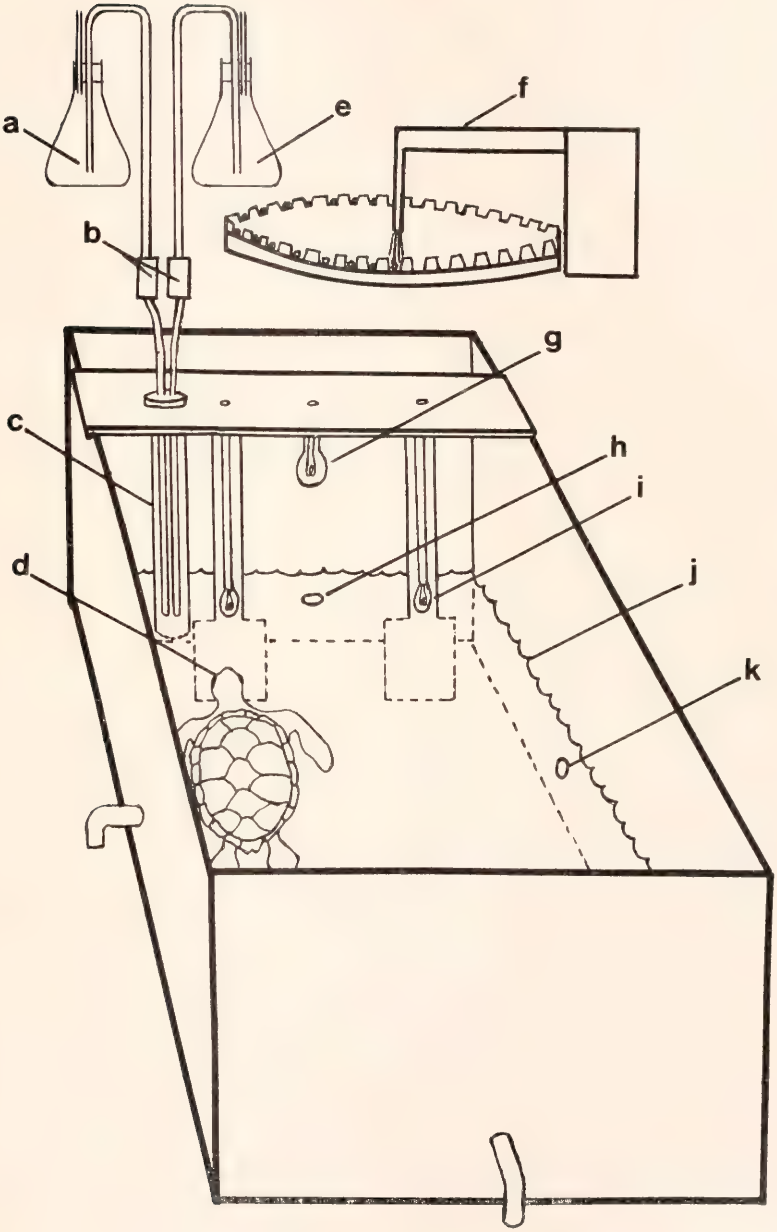

FIGURE 1. Diagram of the experimental tank; (a) chemical or water reservoir; (b) release valves; (c) glass conduit housing delivery tubes; (d) turtle pressing left (signal production) key; (e) second reservoir; (f) automatic feeder; (g) overhead light; (h) water inlet; (i) key light; (j) water level; (k) one of the three water outlets.

tank at a controlled temperature of 26° C and a rate of 8 l/min. The turtles were able to swim about freely, with their heads submerged. Two response keys 4 × 5 cm in size and 8 cm apart were suspended under water at one end of the tank in the path of the water inflow. A light was mounted above water level over each key. An overhead light provided general tank illumination. An automatic feeder was positioned over the right key. The experiment was programmed by standard relay equipment housed in a separate room. Data were recorded automatically on counters. A masking noise was provided in the experimental room to eliminate possible cues from apparatus sounds.

The turtles were trained in a series of steps, using techniques modified from Nevin (1970) and described in detail elsewhere (Manton, Karr and Ehrenfeld, in press). Briefly, the turtles were acclimatized to the tank until food was accepted, then they were required to press the right key with their heads in order to obtain a food reward (small cubes of meat, which were dropped automatically into the tank). Subsequently they were trained to press first the left key, then the right key before reinforcement was delivered. During this initial training a light signal was the cue for reinforcement availability. When this task was learned the light was illuminated on an intermittent schedule. Soon the turtles were observed to press the left key almost continuously, while watching the light over that key. After this light was turned on, if the turtle then responded to the right key within 20 sec, reinforcement was delivered. After each correct response to the right key the light above that key was illuminated for 1 sec. This light acted as a secondary reinforcer, signalling the delivery of food to the tank.

When the turtles had acquired stable behavior patterns and correct responding was at the 90% level, the intensity of the signal light was progressively reduced to zero and the chemical signal was simultaneously introduced in its place. Thus, in the final procedure, the turtles worked steadily at pressing the left key. Whenever a response at this key resulted in the underwater release of chemical, the turtle was given 20 sec in which to press the right key for a food reward.

Although the use of two underwater keys complicated the training procedure, responses to the left (signal production) key maintained attention to the stimulus and insured that the turtle's head would always be near the chemical release point upon signal presentation. The right (reinforcement) key served as the reporting key when a stimulus was detected. The tendency to respond occasionally to this right key when no stimulus was present was reduced by the necessity of activity at the left key, and was further discouraged by the introduction of a 2-sec blackout period (mild negative reinforcement) following each false report.

After training, each experimental session consisted of 50 trials, 25 with 0.5 ml of the test chemical and 25 with 0.5 ml of a water control, presented in a quasi-random sequence. Two separate delivery systems released the test liquids to the tank underwater. These systems were constructed of glass tubing; solenoid-operated valves controlled the flow of the contents of the two delivery tubes. Both tubes released their contents into a common conduit which extended just below the surface of the water near the left hand response key (see Fig. 1). The test liquids were occasionally alternated between the two delivery systems.

The first response to the left key after a minimum time of 1 min had elapsed from the previous trial, started a new trial. During the 20 sec after chemical presentation, a response to the right key was scored as a correct report and reinforcement was delivered. During the 20 sec after water presentation, a response to the right key was scored as a false report and no reinforcement was delivered. The false reports were used as controls to sample the tendency to respond to the reinforcement key when no signal was present.

The first test substance was 0.05 M β-phenethylalcohol. This chemical has been used in olfactory threshold studies with teleost fish (Teichmann, 1959) and is non-toxic, non-irritating and colorless at the concentration used. The interval between chemical trials varied from 1 to 4 min during a session. A minimum interval of 1 min was chosen because tests with an indicator dye added to the tank water in the same concentration as the test chemical, showed a 95% reduction from the initial concentration during the first min after dye addition.

The other organic chemicals tested were also selected on the basis of their use in previous experiments on chemoreception. They were, in order of their presentation: 0.05 M iso-pentyl acetate, 0.01 M triethylamine, 0.01 M cinnamaldehyde, 0.1 M L-serine and 0.1 M glycine. (Calculations of actual concentrations in the tank at the time of chemoreception are presented in "Discussion.") The procedure was identical in all cases. Data were collected from a minimum of 10 consecutive sessions for each test chemical.

The experiment, as outlined, cannot differentiate chemical discrimination mediated by olfaction from that mediated by taste. Therefore the method developed by Alberts and Galef (1971) for producing temporary anosmia in rats by bathing the olfactory mucosa with $ZnSO_4$ solution, was modified for use with these marine turtles. Reagent grade $ZnSO_4 \cdot 7H_2O$ was used in making solutions. After testing several concentrations we selected 0.35 M $ZnSO_4$ for use.

Before treatment with either $ZnSO_4$ or a control solution (NaCl or $MgSO_4$) each turtle was removed from the home tank for at least one hour to permit drying of the nasal cavities and mouth. The turtle was then placed on its carapace with its head tilted downwards. The mouth was held open, and the tongue was screened from contact with the $ZnSO_4$ during treatment. The solution was injected, using a recurved and blunted syringe needle, directly into the internal nares. Approximately 0.3 cc was introduced on each side; drops were observed to run out of the external nares. The area around the internal nares was aspirated to remove any excess $ZnSO_4$ and the turtle was kept in the same position for a few minutes to prevent solution from draining back into the mouth. The turtle was returned to the home tank an hour after treatment. Two turtles (Nos. 2 and 3) were treated with 0.35 M $ZnSO_4$ solution in this manner. The other pair received an identical intranasal injection of either 0.35 M NaCl (No. 1) or 0.35 M $MgSO_4$ (No. 4) as controls.

Turtles were run in their usual chemical discrimination test sessions on the same day as treatment and daily thereafter until behavior returned to the pretreatment baseline.

The treatment was then repeated with the modification that the intranasal injection was made through the external nares. Care was taken to ensure that the head tilted downwards throughout the treatment to minimize flow to the mouth; the mouth was opened, and the solution was injected until it welled up in the internal nares and in the nostril not being injected. The previous control animals

Table I

Mean responses (%) to 4 chemicals and to water, averaged over 15 sessions

Turtle No.	β-phenethylalcohol		iso-pentyl acetate		triethylamine		cinnamaldehyde	
	Mean correct detection (%)	Mean false reports (%)	Mean correct detection (%)	Mean false reports (%)	Mean correct detection (%)	Mean false reports (%)	Mean correct detection (%)	Mean false reports (%)
1	75.7	32.3	86.1	45.3	87.5	42.1	81.6	28.5
2	93.1	42.1	96.3	65.6	94.9	57.3	94.4	47.2
3	85.9	23.2	92.3	52.5	95.7	49.6	92.5	45.9
4	77.3	14.4	85.3	39.2	93.1	44.0	92.0	37.1
Average	83.0	28.0	90.0	50.7	92.8	48.3	90.1	39.7

were injected with $ZnSO_4$ solution (Nos. 1 and 4) while the other pair, which had recovered full olfactory function, received identical injections of NaCl (No. 2) and $MgSO_4$ (No. 3).

Results

Once trained, the green turtles maintained a steady base rate of response to the left (signal production) key with an average of 10 responses/min. Nevertheless, the tendency to respond occasionally to the food key when no signal was present was never completely eliminated.

Approximately 30 sessions/turtle were required to effect the transfer of the learned operant behavior from the progressively reduced light signal to the first chemical signal employed (0.05 M β-phenethylalcohol). By the time the light was dimmed to almost zero intensity the turtles had ceased to look at it, as was their previous habit. The results from 15 consecutive sessions with phenethylalcohol after signal pairing was discontinued are graphed in Figure 2A. The open circles of each graph show the % correct reports after chemical release and the open squares show the % false reports after water release. The turtles all responded to the right (food) key in the presence of the phenethylalcohol solution with a consistently higher probability than to the water control. The mean performance for the phenethylalcohol trials for all 4 turtles, averaged over the 15 sessions (a total of 1500 trials), was 83% correct detection. The mean performance for the same number of water control trials during the same 15 sessions was 28% false reports. The individual means are included in Table I.

Similar results were obtained when the test chemicals were 0.05 M isopentyl acetate, 0.01 M triethylamine and 0.01 M cinnamaldehyde. In these cases, the turtles readily generalized the experimental function of "chemical" and retraining was not necessary when a new test substance was introduced. The per cent correct detection of the test chemical and the per cent false reports to the water tended to vary together. This covariance indicates that although absolute detection varied, relative detection was fairly stable. This was largely a function of a turtle's general activity level on a particular day.

Table I summarizes the data for the first 4 chemicals tested. Each entry in Table I reports the per cent response by an individual turtle to 375 chemical or water trials during 15 consecutive sessions.

TABLE II

Mean responses (%) to 2 amino acids and to water, averaged over 10 sessions

Turtle No.	L-Serine		Glycine	
	Mean correct detection (%)	Mean false reports (%)	Mean correct detection (%)	Mean false reports (%)
1	65.7	71.6	80.8	80.0
2	84.4	84.8	85.6	86.0
3	85.2	82.4	86.0	84.6
4	74.8	75.2	79.2	68.0
Average	77.5	78.5	82.9	79.7

Direct observation of the turtles during their daily sessions revealed a distinctive change in their otherwise leisurely behavior, upon release of the chemical. Flipper movements markedly increased and approaches to the reinforcement key were often quite violent. At times, after chemical release, the turtles became too excited to push the key within the 20 sec limit allotted for correct detection. This frenzied behavior was not observed during water trials. The entire behavioral sequence of pressing on the left key, swimming over to the right key and pressing it, invariably occurred with the head completely under water. During chemical release the turtles directed their nostrils downward and appeared to be pumping water through the nasal cavities by means of throat movements. Breathing pauses, during which the nostrils were above water, were infrequent and were only made during well defined breaks in responding.

As a session progressed we were able to detect a slight odor of the test chemical above the experimental tank. However, it appears virtually impossible that the turtles were using this odor as a cue since the correlation between airborne odors, reinforcement availability and the emergence of the nostrils above water was necessarily random.

The results of the tests of the two amino acids, L-serine and glycine, are given in Table II, and the L-serine results are graphed in Figure 2B. Trials were stopped after 10 consecutive sessions because the learned response pattern was disintegrating in the absence of any stimulus that the turtles could discriminate. It was necessary to retrain the turtles to baseline performance after each amino acid was tested. Phenethylalcohol, earlier shown to be detected, was used for this purpose.

$ZnSO_4$ solution temporarily interrupted chemical discrimination for periods lasting from 1 to 5 days. Recovery of function occurred gradually over a period of several days. Chemoreception before and after $ZnSO_4$ treatment can be compared for all 4 turtles in Figure 3A. Turtles 1 and 4 received $ZnSO_4$ through the external nares and the resulting anosmia was brief. The intranasal injections of saline (turtles 1 and 2) and $MgSO_4$ solution (turtles 3 and 4) had virtually no effect on the performance of the chemical discrimination, as shown in Figure 3B. None of these localized chemical treatments caused any other observed behavioral changes. Turtles treated with $ZnSO_4$ swam and fed normally immediately after treatment. The discriminative test chemical was 0.01 M cinnamaldehyde.

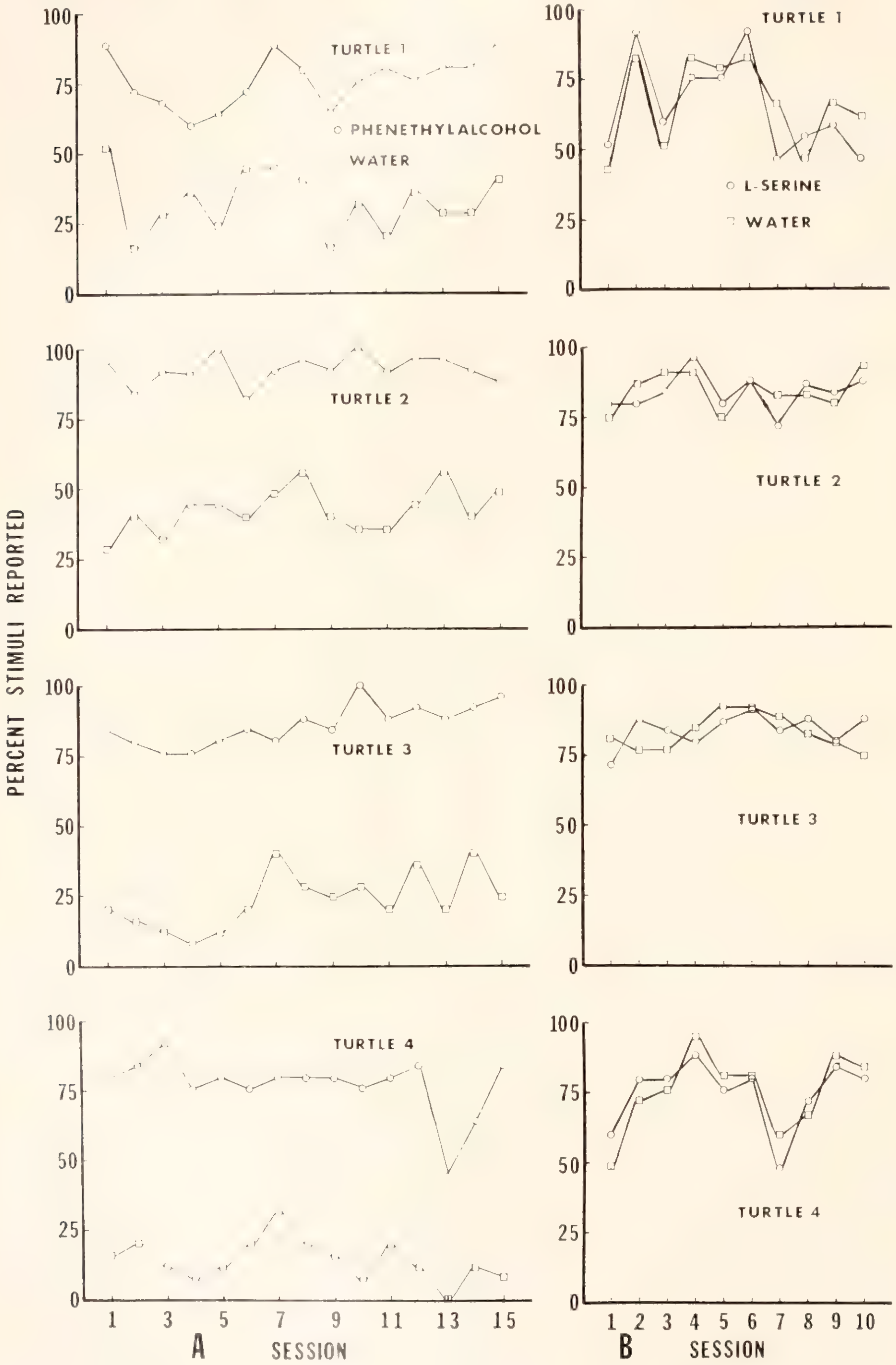

FIGURE 2. The per cent correct and false reports for 4 green turtles during presentation of phenethylalcohol (A) and L-serine (B). The open circles show the per cent correct reports after chemical release. The open squares show the per cent false reports after water release.

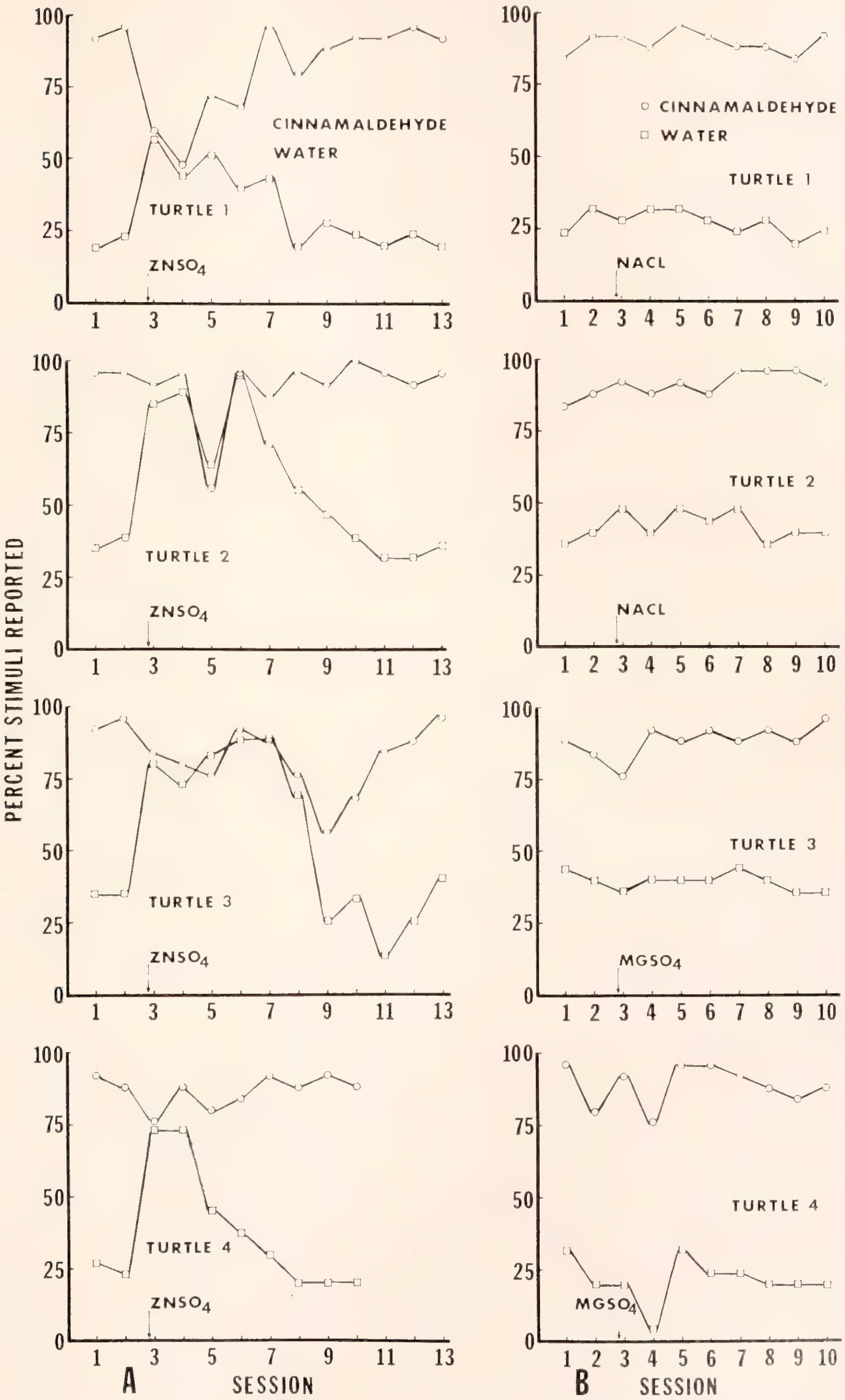

FIGURE 3. The per cent correct (open circles) and false (open squares) reports for 4 green turtles before and after intranasal treatment (arrow) with $ZnSO_4$ (A) or either NaCl or $MgSO_4$ (B). See text for further details.

Discussion

Our results indicate that green turtles can detect a chemical dissolved in water. The inability of the turtles to perform the discrimination following treatment with $ZnSO_4$ solution suggests that this chemoreception is mediated by olfaction. Thus these turtles are able to smell underwater, an unusual ability for an air-breathing vertebrate (Evans and Bastian, 1969). [For purposes of this discussion we assume that "olfaction" is mediated by the entire sensory epithelium of the nasal cavity, which includes some tissue possibly homologous with Jacobson's organ of other reptiles, and which is innervated by the vomeronasal nerve (Parsons, 1967).]

There was no evidence that taste played a role in the performance of the chemical discriminations, although further study is indicated. In histological examinations of the epithelia of the palate and tongue of *Chelonia,* we have so far been unable to identify taste receptors, whereas anatomical structures associated with olfaction are present and well developed. In other turtles (the land tortoise, *Gopherus*), taste receptors have been found to be small and localized to the tip of the tongue (personal communication, P. P. C. Graziadei, Department of Biological Sciences, Florida State University).

Sensory adaptation has not proven to be a problem in our procedure. Although the test chemical was not completely cleared between all trials, a sudden increase in chemical concentration was satisfactory as a discriminative stimulus. If some sensory adaptation did occur between trials, despite steadily decreasing concentration, the addition of the test chemical solution at the next trial provided a sufficient change in the stimulus to act as a new signal. This was clearly confirmed both by the test scores, themselves, and by the directly observed behavioral changes which occurred after presentation of chemical stimuli.

The sound of running water served to mask the noise of the solenoid-operated valves, and the control and chemical solutions were occasionally switched in any case so that no valve sound could serve as a reliable cue to chemical presentation. Relays, counters and tape programming apparatus were all located in an adjacent room behind a closed door, and their various sounds (similar for chemical and water trials) were inaudible to us in the room with the test chamber. We have dismissed the possibility that inadvertant apparatus sounds could function in the discrimination.

Differences among the average scores for the first 4 chemicals tested, and for their controls (Table I), probably represent differences in the turtles' level of training and experience and in their strategy of response rather than differences in perception of the various odors. In each of these test series the fact that there is a large and consistent difference between correct detection and false reports is more significant than the exact amount of that difference.

A fairly high rate of inappropriate responding to the reinforcement key in the absence of a stimulus is common in operant situations where there is little or no effective punishment for making false responses (Azrin and Holz, 1966). The 2-sec blackout did not constitute strong negative reinforcement. (During training, when the light was the discriminative stimulus, the percentages of both false and correct reports were comparable to those recorded in the chemical trials.)

The transfer from one test chemical to another presented no difficulties and gave clear evidence of stimulus generalization. A single session was usually sufficient to establish correct responding to the new odor. On the other hand, when the stimulus presented could not be detected, behavior was disrupted. The behavior during the amino acid test sessions was characterized by alternation between the keys, frequent pauses between bouts of responding and occasional defecation in the experimental tank. (Salmon, unlike *Chelonia*, are reported to be able to detect L-serine in extremely low concentrations (Idler, Fagerlund and Mayoh, 1956).)

It is possible to make an estimate of the sensory acuity demonstrated in this experiment. From the relative positions of the turtle (at the left key) and the chemical release point we can assume that the delivered chemical is diluted, at the time of sampling, in a volume of water equivalent to ¼ of the tank volume (1000 ml). Detection therefore occurs at approximate concentrations of from 5×10^{-6} M to 5×10^{-5} M depending on the solution used. Dye tests confirm the assumption underlying this calculation. (The undetected amino acids were presented at an approximate concentration of 10^{-4} M.)

The mechanism of the $ZnSO_4$-induced anosmia is as yet unknown. The data from the saline and $MgSO_4$-treated controls appear to rule out osmotic shock or trauma following treatment. The role of Zn^{++} seems crucial. The present method of $ZnSO_4$ application produces considerable variation in the period of anosmia. One turtle showed definite signs of the return of olfaction after 24 hr, while the longest period of complete anosmia was 5 days. Factors such as the mode of administration and the degree of dryness of the nasal passages probably influenced the effectiveness of the treatment.

Since the animals used in this study are difficult to obtain, Zn^{++}-induced peripheral anosmia has certain advantages over olfactory bulb ablation or olfactory nerve sectioning. The Zn^{++} effect is both reversible and relatively non-traumatic. Furthermore, the present technique provides the opportunity for field studies of the role of olfaction in the orientation of green turtles, without causing permanent loss of functional individuals from an endangered population. The possible role of olfaction in both open sea navigation and in site selection at the nesting beach can be experimentally studied with this approach. In general, the use of Zn^{++}-induced anosmia offers promise of opening new areas of investigation of the interaction between olfaction and behavior among a wide range of vertebrates; and it may provide an additional tool for the study of the mechanism of olfaction itself.

Our experimental procedure is a sensitive behavioral assay for underwater chemoreception in aquatic vertebrates. It further demonstrates the utility of operant conditioning methods in the study of reptilian sensory physiology. Our findings show that the migratory sea turtle, *Chelonia mydas*, can smell a variety of chemicals dissolved in water in moderately low concentrations. Such detection is a prerequisite sensory capability if, as has been suggested, chemical cues borne in ocean currents play a role in navigation.

We thank Drs. A. Carr, J. E. Bardach, D. R. Griffin and J. A. Nevin for encouragement and advice. The Caribbean Conservation Corporation aided us in the collection of green turtle eggs. This work was supported in part by Barnard

College faculty research grants and a grant from the Penrose Fund of the American Philosophical Society (to D. W. E.).

SUMMARY AND CONCLUSIONS

1. The ability of the green turtle (*Chelonia mydas*) to detect various chemical substances dissolved in water has been investigated using operant conditioning techniques. The turtles pressed underwater keys to obtain food reinforcement in the presence of a chemical stimulus.

2. The turtles were capable of underwater chemoreception of β-phenethylalcohol, iso-pentyl acetate, triethylamine and cinnamaldehyde at approximate concentrations of 5×10^{-6} M or 5×10^{-5} M, but not of L-serine or glycine at an approximate concentration of 10^{-4} M.

3. Stimulus generalization occurred when turtles were shifted from one test chemical to another.

4. Intranasal injection of 0.35 M zinc sulfate solution interrupted olfaction for periods of from 1 to 5 days. Treatment with 0.35 M saline or magnesium sulfate had no effect on the performance of the chemical discrimination. It was concluded on the basis of these experiments that chemoreception in *Chelonia* is largely or entirely mediated by olfaction rather than by taste.

5. The advantages of the zinc-induced anosmia over surgical techniques and the possible use of the zinc treatment in field studies of orientation are discussed.

6. Our results provide evidence to support the current theory that soluble compounds entering ocean currents from the vicinity of nesting sites might be detected by green turtles, and that this could aid in navigation.

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CIRCADIAN RHYTHMS: MECHANISM OF LUCIFERASE ACTIVITY CHANGES IN *GONYAULAX*

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Gonyaulax polyedra is a bioluminescent dinoflagellate which exhibits a daily rhythmic fluctuation in its capacity for bioluminescence (Hastings and Sweeney, 1958). When grown using a LD 12:12 cycle (12 hours light alternating with 12 hours darkness), the period of the bioluminescence rhythm is exactly 24 hours; the maximum luminescence is in the middle of the dark period. However, rhythmicity will persist in constant laboratory conditions, where it assumes its "circadian" period, close to but not exactly 24 hours. Under these conditions the *phase* of the rhythm is independent of that of the earth's daily cycle (Aschoff, 1956; Bünning, 1967).

Little is known about the mechanism of circadian rhythms or about the biochemistry involved in their expression.

In *Gonyaulax* activity of the extractable soluble enzyme luciferase displays a circadian rhythm which correlates well with the bioluminescence rhythm of the intact cell. Luciferase, which catalyzes *in vitro* luminescence via oxidation of a low molecular weight substrate "*Gonyaulax* luciferin," has been partly purified and characterized (Bode and Hastings, 1963; Fogel and Hastings, 1971). The luciferase activity in the supernatant resulting from centrifuging a cell homogenate for about 30 minutes at 27,000 × *g* fluctuates rhythmically with time of extraction, both for cells from a light-dark cycle (Hastings and Bode, 1962) and for cells from constant dim light (Fig. 1). Hastings and Bode (1962) reported that this rhythm was not one of total protein extractability and that the day-night difference in luciferase activity per mg protein was retained after an 8-fold purification.

A number of other cases in which enzymatic activity varies from night to day are known (Sanwal and Krishnan, 1960; Potter, Gebert, Pitot, Peraino, Lamar, Lesher, and Morris, 1966; Rapoport, 1966; Civen, Ulrich, Trimmer, and Brown, 1967; Hardeland, 1969; Sweeney, 1969), but in only a few cases (Hardeland, Sweeney) has the molecular basis been examined, and in no case has it been well defined.

We report here further experiments to discover the immediate biochemical basis for the cycling in *Gonyaulax* luciferase activity. We also discuss the contribution of the luciferase rhythm to the bioluminescence rhythm of the intact cell.

MATERIALS AND METHODS

Gonyaulax polyedra is a photosynthetic, bioluminescent, armored marine dinoflagellate about 40 μ in diameter. Two strains were used. The non-axenic strain was that used by DeSa (1964) and reported by him to have been isolated in

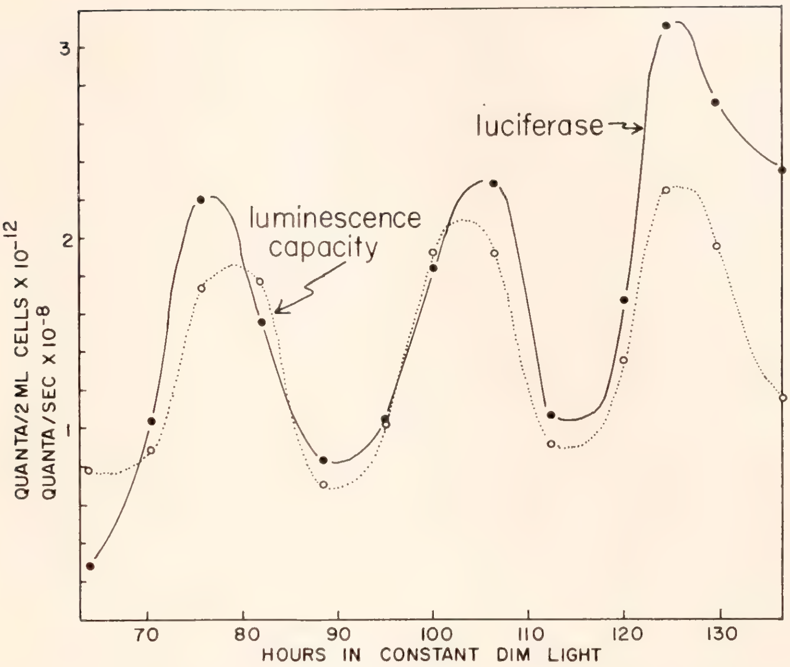

FIGURE 1. Luciferase activity rhythm and luminescence capacity rhythm in constant light (160 footcandles), 22° C. The culture was transferred into constant light at the end of a light period. Cells were pelleted from 80 ml of culture by a 1.5 minute centrifugation at speed 3 in an International clinical centrifuge (more convenient for harvesting small volumes of culture than filtration through a Büchner funnel), suspended in 16 ml 0.05 M tris, 0.01 M EDTA, pH 8, 0.005 M DTT, and homogenized once. The cell debris was removed by 5 minutes' centrifugation at 2000 × *g* and reextracted with 8 ml. The first supernatant and second homogenate were combined and centrifuged 15 min at 27,000 × *g*. The supernatant was assayed for luciferase in assay mixture #1.

1952 by Dr. B. M. Sweeney. Cultures were maintained as previously described (Fogel and Hastings, 1971). The second strain (used only for the experiment shown in Figure 1) was an axenic clone derived in the laboratory of Dr. Robert Guillard, Woods Hole Oceanographic Institute, from a strain isolated by Dr. Sweeney in 1960. The data in Figure 1 accompanied other measurements, not pertinent here, which required an axenic culture. The second strain was maintained in "f/2" medium ("f/2" medium is the "f" medium of Guillard and Ryther (1962) diluted in half with sea water). Both strains have similar luciferase rhythms.

Luminescence was measured by a photomultiplier and amplifier as described by DeSa (1964). The instrument was calibrated at 490 nm using the secondary luminescence standards of Hastings and Weber (1963). The intensity of fluorescent lights used in growing cells was measured with a Weston illumination meter model 756, quartz filter.

Under LD 12:12 growth conditions, we found the luciferase activity of "midnight" extracts (made after 5 to 7 hours of darkness) to be about 10 times that of "mid-day" extracts (made after 5 to 7 hours of light), while under constant light the rhythm had a smaller amplitude (Fig. 1). Therefore we used LD conditions and mid-day and mid-night extraction times to assure both a large difference between minimum and maximum luciferase activities and predictability for the phase of the rhythm. A culture of 3000 to 10,000 cells/ml (uniform in any given experiment) was harvested by filtration on a Büchner funnel (except in Fig. 1) and the cells were suspended in either cold pH 6.0 extraction buffer (0.05 M sodium potassium phosphate) or cold pH 8.0 extraction buffer (0.05 M tris, 0.01 M EDTA) with 0.001 to 0.005 M DTT. Extraction in the first buffer yields luciferase of approximately 35,000 molecular weight, the second 150,000 M.W. (Fogel and Hastings, 1971). Ten ml or more of extraction buffer was used per 800 ml of culture. The cell suspension was passed twice through a stainless steel hand emulsifier (Fisher Scientific Co., catalog #11–504–2000). The homogenate was centrifuged in the HB4 swinging bucket rotor in a Sorvall refrigerated centrifuge as described in the figure legends and the pellet discarded.

In a study of luciferase extractability, guanidine was used in the extraction medium. Cells were extracted by homogenization in 5 M guanidine hydrochloride, 0.005 M DTT, pH 6.7 at 22° C. As the control, cells were extracted with the pH 6 extraction buffer at 4° C. "Day" cells were harvested 5.5 hours after lights on and "night" cells 4.5 hours after lights off. The crude homogenate was centrifuged at 25,000 × *g* for 10 minutes to give a supernatant. The luciferase, denatured by guanidine, was renatured by 1/25 dilution into 0.05 M tris, 0.01 M EDTA, 0.001 M DTT, 0.1 mg/ml BSA, pH 8.0 at 4° C, where complete recovery took about 5 hours. Control samples were similarly diluted. Assays were done in assay mixture #2.

To obtain luciferin for the luciferase assay, cultures from the day, or from the night after an hour's exposure to bright light (Bode, DeSa and Hastings, 1963), were harvested as decribed above and cells suspended in buffer in a boiling water bath. The buffer was 0.0025 M tris, 0.0005 M EDTA, pH 8.0; about 1.5 ml per flask of culture (800 ml) was used. After 2 minutes, the solution was chilled, made 0.005 M in DTT, centrifuged 30 minutes at 27,000 × *g*, and the supernatant frozen and stored in 1 ml portions at −57° C.

Two different reaction mixtures were used for the luciferase assay, using a volume of 2 ml in both cases. Assay mixture #1 (Bode and Hastings, 1963) was 1 M ammonium sulfate, 0.1 M tris-maleate, 0.4 mg/ml BSA, 0.0025 M EDTA, pH 6.4 to 6.7. Assay mixture #2 (Fogel and Hastings, 1971) was simpler and so preferred in later experiments; it was 0.2 M sodium phosphate, 0.1 mg/ml BSA, pH 6.2. The assay was carried out by adding (in either order) luciferin and luciferase in prompt sequence; the reaction was initiated by the last addition. The intensity was recorded at a fixed time after initiation (about 10 sec) and was proportional to the luciferase concentration over the range assayed. Light emission without added luciferin was negligible. Unless otherwise specified, the assays were done in duplicate or triplicate with an average error of 11%.

Luminescence capacity refers to the amount of light emitted by the intact cell when stimulated by mechanical or chemical means. In these experiments, two ml of cell culture was placed in a 20 ml vial above a photomultiplier and 1 ml 0.06 N acetic acid was injected into the vial. The burst of light thus elicited was integrated electronically for 5 sec, at which time emission was complete. Assays were done in triplicate and had an average error of 4%.

The activity of luciferases from "day" and "night" cells were compared after centrifugation in sucrose density gradients. Pig heart lactate dehydrogenase purchased from Sigma Chemical Corporation was used as a marker enzyme to control for variations in sedimentation velocity from one gradient to the next. One hundred μl of luciferase from a pH 8 tris extraction was mixed with 10 μl lactate dehydrogenase (0.21 mg/ml) and layered on top of a 4.5 ml, 5–16% convex exponential sucrose gradient, made with 0.05 M tris, 0.01 M EDTA, 0.005 M DTT, pH 8.0. Gradients were spun 23.9 hours at 37,400 rpm in a SW 39 rotor in a Beckman ultracentrifuge model L2. Gradient tubes were impaled on a syringe needle and fractions of 10 drops were collected in small iced tubes containing 25 μl 0.05 M tris, 0.01 M EDTA, 5% sucrose, 0.1 mg/ml BSA. Two luciferase assays were done on each fraction; 50 μl of the fraction was used for each. Assay mixture #2 was used. To assay for lactate dehydrogenase, sodium pyruvate to make 0.00076 M and NADH to make 0.058 mg/ml were freshly added to 0.03 M Na_2HPO_4, pH 7.4; then to 1 ml of this in a cuvette was added a 25 μl gradient sample. The contents of the cuvette were mixed and the change in optical density at 340 nm per minute was measured on the 0.1 slideware of a Cary 15 recording spectrophotometer.

Abbreviations used are: BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; LD 12:12, a light/dark cycle with 12 hours of light alternating with 12 hours of darkness; NADH, reduced nicotinamide adenine dinucleotide; and tris, tris-(hydroxymethyl)-aminomethane.

RESULTS

A trivial explanation for the luciferase activity rhythm, namely, that total protein might manifest such a rhythm in extractability, had been stated to be untrue (Hastings and Bode, 1962). Any individual enzyme such as luciferase, however, might still be bound more securely than proteins generally during the day than during the night and so be retained better by the cell debris. We made a preliminary check for such selective retention by assaying the crude cell homogenate (which includes the cell debris) for luciferase in the standard luciferase assay. Extractions made in pH 6.0 phosphate buffer showed that there was 2 to 3 times more luciferase activity in a crude cell homogenate than in the supernatant after the cell debris had been removed by centrifugation. This difference, however, was found in extracts made during the night as well as during the day. These measurements therefore gave no evidence that selective retention of assayable luciferase by cell debris during the day explained the luciferase rhythm.

The possibility remained that only a fraction (say 10%) of the luciferase in "day" cells was similar in extractability and activity to that in "night" cells while the bulk of luciferase in day cells was rendered both inextractable and inactive by its location within the cell. Were this so, the luciferase might become assayable if it could be released from its location. Therefore we tried different mechanical methods of extraction as well as different extraction media in an effort to bring the activity of day extracts up to the level of that of night extracts.

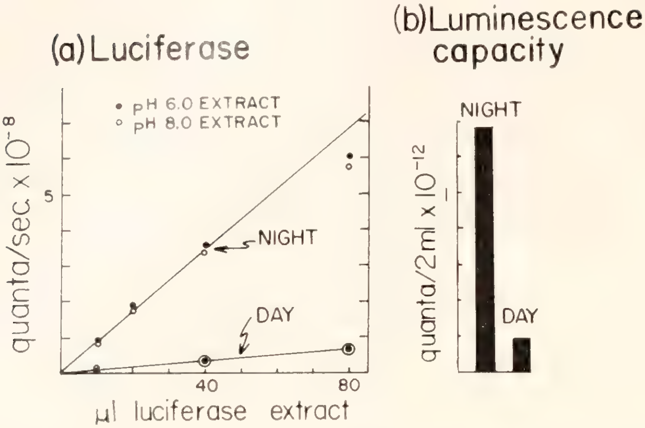

FIGURE 2. (a) Comparison of luciferase activity in extracts made in 0.05 M phosphate buffer, pH 6 ("pH 6 extract") and in 0.05 M tris, 0.01 M EDTA, pH 8 ("pH 8 extract") during both the day and the night. Fifteen hours before the first harvest, cultures of cells were combined and divided between two flasks. Extractions were made with 0.001 M DTT 7 hours after lights off (NIGHT) and 3.5 hours after lights on (DAY), using one flask for each time, one half of the flask for each of the two extraction pH's. The extract was spun 3 min at 2,000 × g to remove cell debris, then 15 min at 20,000 × g. Assay mixture #1 was used, final pH 6.7, one assay per point. (b) Luminescence capacity measured in the same experiment just prior to cell harvest.

In all cases we failed to accomplish this. Typical day-night differences in luciferase activity were observed whether the extraction was done by stirring the cells in buffer or by emulsification with the Fisher emulsifier. A Ten-Broeck glass homogenizer was found to be only about half as effective as the emulsifier in releasing luciferase during *both* the day and the night. In addition, we found similar activities in extracts made in phosphate buffer at pH 6 and in tris buffer with EDTA at pH 8 at any given time, while the day activity was still about 10% of the night activity (Fig. 2). These findings are also of interest because it had been shown that the extraction medium determines which of two molecular weight forms of luciferase would be obtained, 35,000 (phosphate buffer, pH 6) or 150,000 (tris buffer with EDTA, pH 8) (Fogel and Hastings, 1971).

Another variation in the extraction medium involved the use of guanidine, which disrupts noncovalent bonds (which might be responsible for holding day luciferase more firmly in the cell) (Tanford, Kawahara, Lapanje, Hooker, Zarlengo, Salahuddin, Aune, and Takagi, 1967). Extraction in the emulsifier with 5 M guanidine did not release proportionately more luciferase activity from "day" cells than it did from "night" cells in comparison to pH 6 phosphate extraction buffer. (See Methods for details. The luciferase extracted in guanidine is denatured but is restored to complete activity by subsequent dilution in tris buffer at pH 8.) Extraction in quanidine apparently detached all the assayable luciferase from the cell debris, since the same recoverable activity was found in the guanidine supernatant above the cell debris as was found in the guanidine (and phosphate) crude homogenates before the cell debris was removed by centrifugation. Extraction in

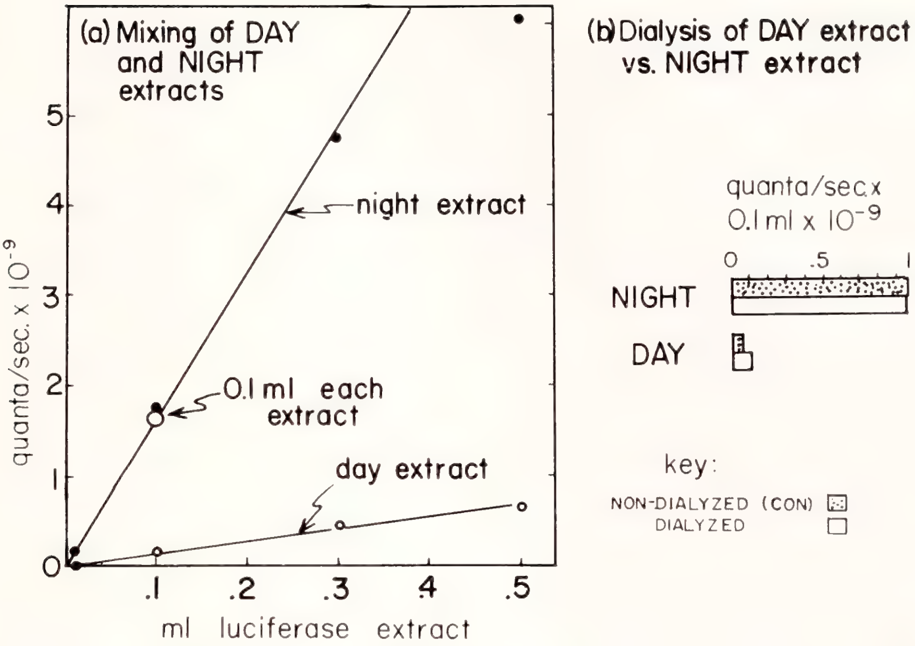

FIGURE 3. Two experiments testing for presence of an inhibitor or activator of luciferase in extracts made during the day and during the night. Five days before harvest time a flask from constant dim light was diluted with new medium; half these cells were put on one LD 12:12 cycle, the other half on the same cycle, 12 hours out of phase. Cells were harvested 6 hours after lights on or lights off and extracted in 0.05 M tris, 0.01 M EDTA, pH 8.0 (0.005 M DTT). The homogenate was centrifuged 1 hour at $23{,}000 \times g$. (a) Mixing aliquots of day and night extracts to check whether activities were additive. $(0.5-x)$ ml extraction buffer and x ml luciferase extract were added to assay mixture #1 containing 50 μl luciferin. (b) Dialysis of day extract against night extract. 1.5 ml day extract was put inside a dialysis bag and dialyzed 13 hours at 4° against 3 ml night extract; activity changes were compared to those of undialysed extracts containing pieces of dialysis bag (controls); 0.1 ml luciferase was assayed.

phosphate buffer, on the other hand (as mentioned above), apparently detached only $\frac{1}{3}$ to $\frac{1}{2}$ of the available luciferase activity.

From these experiments there is no evidence that the luciferase rhythm is due to a rhythm in luciferase extractability. We now turn our attention to the possible presence of activators or inhibitors in the extracts.

Were an activator present in the night extract in a greater than stoichiometric amount, this extract would enhance the day extract's activity; conversely, a day extract, if it contained an inhibitor, should reduce the activity of the night extract. The total activity of such mixtures of day and night extracts was, however, approximately the sum of that of the two constituents (Fig. 3a). Several other experiments confirmed this; the slight inhibition seen in Figure 3a fell within experimental error.

If such an activator or inhibitor were present but in only stoichiometric amounts, the activity of mixtures *would* be additive. If it were, however, not tightly bound,

TABLE I

Effect of ammonium sulfate precipitation upon the activity of luciferase from the day and from the night. (The same luciferase extract was used as for Figure 3. The supernatant from the 1 hour's centrifugation was made 70% in ammonium sulfate over a 5 minute period and stirred then for 10 minutes at 4° C. The luciferase was pelleted at 15,000 rpm in a Sorvall SS34 in 30 minutes and resuspended in 1 ml extraction buffer. Ten μl of extract was assayed for luciferase in the manner of Figure 3, A.S. = ammonium sulfate precipitation)

Time at which extract was made	Total activity of extract: (quanta/sec)		% Recovery
	Before A.S.	After A.S.	
Day	12×10^9	6.7×10^9	56%
Night	120×10^9	72×10^9	60%

it should be removable upon purification of the enzyme. However, the difference between day and night luciferase activity (for the 150,000 molecular weight form) persisted after dialysis (even when day luciferase was dialyzed against night luciferase (Fig. 3b)), ammonium sulfate precipitation (Table I) and sucrose velocity gradient centrifugation (Fig. 4), each done on crude enzyme. Therefore, either no activator or inhibitor was present, or, if one was, it did not separate from its luciferase during these purification treatments. Separation might have failed to occur if the hypothetical inhibitor or activator were bound to the luciferase or if it behaved similarly to luciferase during purification.

DISCUSSION

Summarizing these experiments, the rhythm of luciferase activity in crude extracts of *Gonyaulax* cells from a LD 12:12 cycle does not appear to result from a rhythm of luciferase extractability. However we cannot completely exclude this possibility (for example, during the day, luciferase might be covalently bound to the cell debris and rendered inactive). Further, it does not appear to be caused by a rhythm in concentration of an activator or inhibitor molecule of any variety present in greater than stoichiometric amount; nor does it appear to be caused by a rhythm in dissociable activator or inhibitor molecule present in stoichiometric amounts unless such a molecule is similar to luciferase in its behavior upon dialysis, ammonium sulfate precipitation, and sucrose velocity gradient centrifugation.

The results thus rule out several explanations for the rhythmic change in luciferase activity. Two possible explanations appear to remain. One is that there are simply more luciferase molecules present in night cells than in day cells. Such a situation would imply large scale *de novo* synthesis and degradation processes *in vivo* not associated with growth. A method similar to that of Filner and Varner (1967) involving heavy isotopes C^{13} and N^{15} and sucrose velocity gradients was used to investigate this possibility (McMurry, 1971) but the results were not conclusive; they suggested that there may not be sufficient luciferase synthesis to support a hypothesis of complete *de novo* synthesis from amino acids.

A second explanation would involve a chemical moiety which attaches and

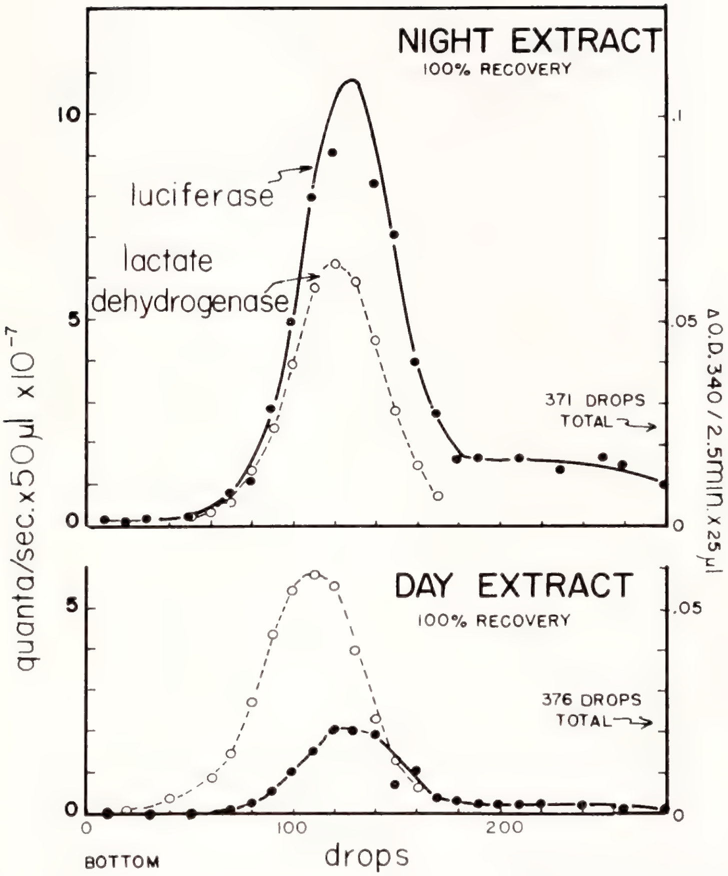

FIGURE 4. Behavior of luciferase activity from a day and a night extract during sedimentation of the extracts through sucrose gradients, showing persistence of the difference between day and night activities. Half of each of two 800 ml cultures was harvested 6 hours after lights on (DAY) and 4.5 hours after lights off (NIGHT), extracted in 0.05 M tris, 0.01 M EDTA, pH 8.0, 0.005 M DTT, centrifuged 40 min at 25,000 × *g*, and stored at −57° C. Gradients were run as described under Methods.

detaches, possibly covalently, to and from the "backbone" of the luciferase molecule during each circadian cycle. The presence of this moiety would alter the luciferase activity. Luciferase would thus occur in two forms, one more active than the other. In the absence of growth, the total number of luciferase "backbone" molecules would remain constant. One of two opposite situations might obtain: (1) the moiety might cause a 10-fold change in activity; every "day" luciferase molecule might then be 10% as active as every "night" molecule, in which case the active species would differ chemically from day to night; (2) the moiety might completely activate or inactivate; all of the molecules would be active at night while only 10% of the molecules would be active by day. In this case the active species would be the same from day to night.

For either situation, the moiety could be of any size or nature compared to the luciferase backbone, with appropriate effects upon the separation of backbone from backbone-plus-moiety during purification (the moiety could even be a cellular organelle). The moiety itself could undergo a daily *de novo* synthesis and destruction or it could be always present in the cell. The moiety could be a protein. It should be noted in this respect that an activity rhythm is seen for both the 150,000 and 35,000 molecular weight forms of luciferase (Fig. 2).

If there is any difference in the molecular weight between active day and night luciferase, it is not large (Fig. 4); the somewhat smaller sedimentation velocity for day luciferase seems to be at least partly a function of its lower activity, for if night enzyme is diluted to comparable activity, it has a similarly smaller sedimentation velocity (McMurry, 1971).

The rhythm of luminescence capacity parallels that of soluble luciferase activity (for example, see Fig. 1 and 2). What is the significance of this fact?

Luminescence capacity can be measured in two ways, by stimulating the cells with acid to emit a burst of light (as in this report), or by bubbling them with air to cause many individual bright flashes (Hastings and Sweeney, 1958). Values obtained with acid are two to three times higher than those obtained by bubbling, but the rhythms are otherwise apparently equivalent (McMurry, 1971); we may therefore in this discussion talk about cell flashes.

Over 95% of the light emitted during bubbling comes from cell flashes (McMurry, 1971). However, luminescent particles, the scintillons (DeSa, Hastings, and Vatter, 1963), rather than soluble luciferase, are believed to be responsible for cell flashes (Hastings, Vergin, and DeSa, 1966; Eckert and Reynolds, 1967; McMurry, 1971; Fogel and Hastings, 1972). Hence the question is likely one of the relationship between the soluble luciferase and the scintillons.

Scintillons can utilize free luciferin and likely also contain luciferase (Fogel, 1970; Fogel and Hastings, 1972). The luciferase extracted in soluble form may be in equilibrium *in vivo* with luciferase on the scintillons, or it may be solubilized from scintillons during extraction. In either case, if the amount of light which scintillons emit *in vivo* were for some reason proportional to their luciferase activity content, the soluble luciferase activity would reflect the luminescence capacity.

This research was supported in part by a National Science Foundation Research Grant GB 16512. Laura McMurry held a USPH Predoctoral Traineeship.

SUMMARY

The bioluminescent marine dinoflagellate *Gonyaulax polyedra* manifests similar circadian rhythms of bioluminescence capacity and extractable luciferase activity, both with maxima during the night phase. The immediate biochemical basis of the luciferase rhythm was investigated, with the following findings:

(1) The rhythm was present no matter which of several mechanical extraction methods and extraction media (including 5 M guanidine) were employed. The rhythm was present even in a crude cell homogenate. Thus the rhythm is likely not one of extractibility unless luciferase is inactivated while being covalently bound to cell debris during day phase.

(2) Mixing experiments, ammonium sulfate precipitation, dialysis, and sucrose velocity gradient centrifugation showed that no dissociable activator or inhibitor of luciferase caused the rhythm.

Two possible hypotheses remain untested: (a) the occurrence of *de novo* luciferase synthesis and destruction, (b) the attachment (perhaps covalent) and detachment of an activity-modifying moiety.

The luminescence capacity rhythm is primarily a rhythm of quantity of light from cell flashes. Cell flashes probably originate from extractable particles termed scintillons which flash during assay. The relationship of the luciferase rhythm to the luminescence capacity rhythm is discussed from this view.

Note added in proof: For recent findings on the luminescence capacity rhythm see R. Christianson and B. M. Sweeney, 1972. Sensitivity to stimulation, a component of the circadian rhythm in luminescence in *Gonyaulax. Plant Physiol.*, **49**: 994–997.

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OBSERVATIONS AND EXPERIMENTS ON METHODS OF FERTILIZATION IN THE CHAETOGNATH *SAGITTA HISPIDA*

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For many years, the only laboratory-maintained chaetognath was *Spadella cephaloptera*, a member of the single benthic genus. Until the development of culture ability with *Sagitta hispida* (Reeve, 1970a), no extensive behavioral observations were possible in the pelagic genera, which are generally considered to constitute the second most important group of marine macroplankton. The process by which fertilization occurs in *Spadella*, involving a reciprocal transference of spermatophores between two individuals, has been extensively documented by Ghirardelli (*e.g.*, 1968). For the other genera, there exist scattered observations of a largely circumstantial nature which support the possibilities of both cross- and self-fertilization. These observations are reviewed in detail by Reeve and Cosper (1972) who noted that Hyman (1959) stated that "Self-fertilization is thus apparently the rule in *Sagitta*," (page 29) although Alvariño (1965) concluded for chaetognaths as a whole that ". . . it is generally accepted that cross-fertilization by copulation is the rule" (page 132). Since both reviewers indicated the dominance of the genus *Sagitta* within the phylum, these opinions are essentially contradictory. Ghirardelli (1968) preferred to conclude that one method does not necessarily exclude the other, even in the same species.

Foremost amongst the evidence for self-fertilization is the direct observation of the migration of spermatozoa from the seminal vesicle forward along the tail and into the seminal receptacle in isolated individuals of the species *Sagitta setosa* (Jägersten, 1940; Ghirardelli, 1968; Dallot, 1968). The last author was able to show that this resulted in the laying of fertile eggs in some cases. The evidence supporting cross-fertilization is based on observations of objects considered to be spermatophores attached to specimens from plankton samples (*e.g.*, Dallot, as reported to Ghirardelli, 1968; David, 1958) and the specialization of structure of seminal vesicles in some species, such as *S. bipunctata* where the seminal vesicle is surmounted by a small cup with "saw teeth" edges. Ghirardelli (1968) believed such structures might serve as copulatory organs whose form fits the female genital orifice of that species only, preventing mating between different species. Further circumstantial evidence for cross-fertilization in pelagic chaetognaths occurs in the observation of Murikami (1959) concerning specimens of *Sagitta crassa* which were found adjacent to each other with heads oriented in the opposite direction, presumably copulating. In addition, the fact that cross-fertilization is normal in the only species to be extensively studied alive (*Spadella cephaloptera*), and exclusive self-fertlization at least on an evolutionary time scale seems highly unlikely, suggests that extensive observation of pelagic species would be necessary before attempting to discount any possibility of cross-fertilization.

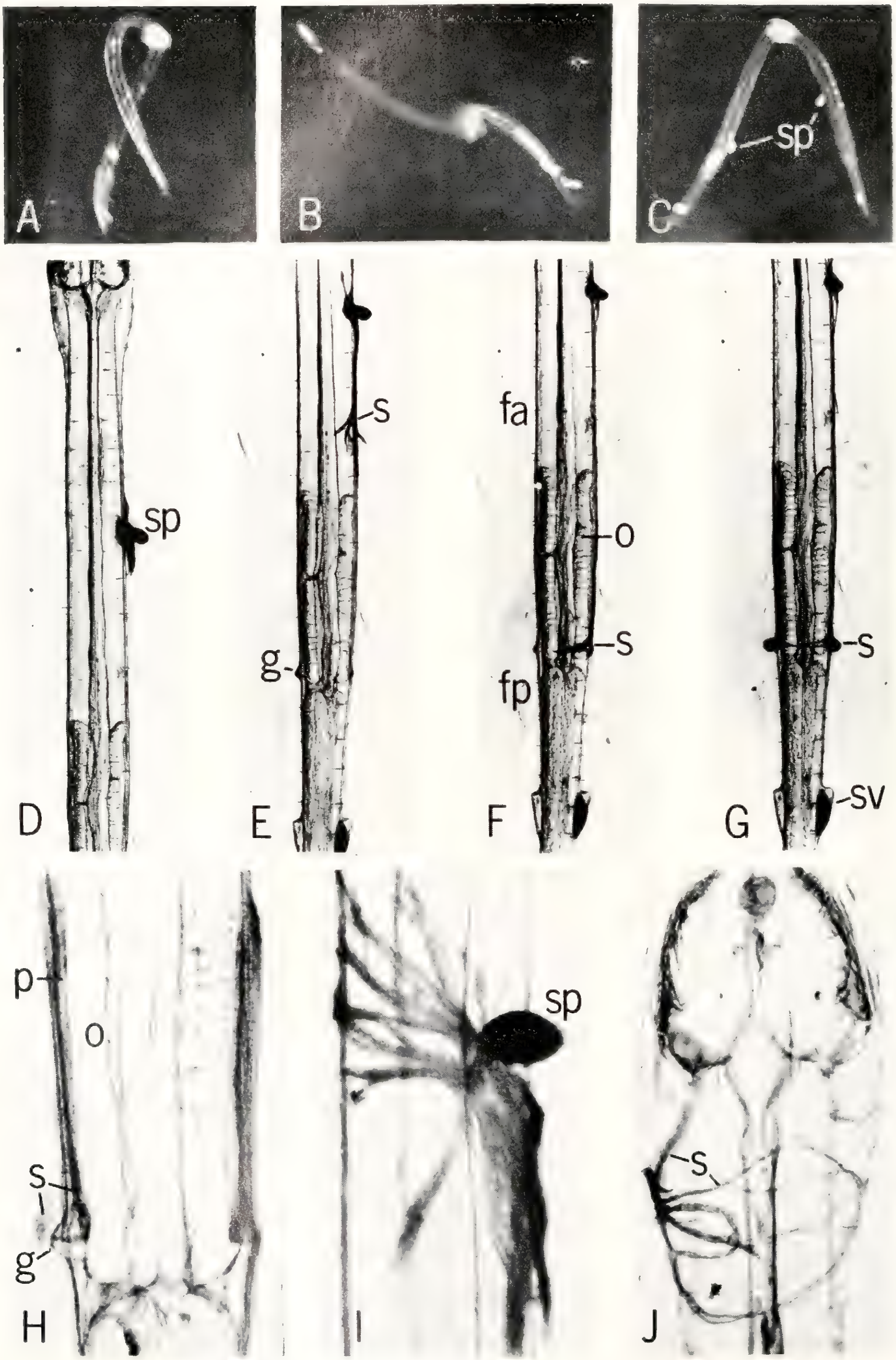
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Figure 1.

The observations and experiments reported below show that both self- and cross-fertilization can occur in the same species and provide some indication of their relative frequency.

Observations and Results

Sagitta hispida Conant occurs in Biscayne Bay, adjacent to the laboratory, and can be maintained successfully in culture (Reeve, 1970a) by providing large aquaria, frequently changed, well aerated water and live zooplankton for food. Observations were made upon populations in rectangular acrylic or glass aquaria in volumes of 40 liters with a water depth exceeding 45 cm. Individuals and pairs were isolated in 250 ml containers.

Cross-fertilization

In laboratory culture acts of cannibalism by *Sagitta hispida* are frequently observed, especially in older animals. Usually the attacker seizes the head of its victim by means of its spines or "jaws" (rather than another part of the body) and proceeds to ingest it whole. When both animals are mature and hence of similar size, several minutes may elapse during which the pair engages in violent swimming motions. Often the animals separate, and swim off apparently unharmed, bearing a small dark attachment on the side of their bodies. Microscopical examination has shown this attachment to be a spermatophore packed with motile sperm.

We have each seen the initiation of attack once. In each case, two animals made several upward darting movements in close proximity to each other before becoming attached. In the first case, spermatophore transference occurred and the animals separated some 90 seconds later. In the second case, they detached within a few seconds without any transference. Rapid darting movements, in which two animals may swim closely together from the bottom to the top of the water column (45 cm) and back again within 5 seconds, are seen mostly in populations in which attachments are occurring. We suggest that it is a behavioral sequence associated with copulation. During the attachment phase of the encounter, the

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FIGURE 1. (A–C) The copulatory "dance." Three separate pairs of animals attached at their head ends engaged in characteristic violent movements during which spermatophores were exchanged. In C, each of the pair has gained a spermatophore. (D–G) External migration of sperm. This sequence taken of a single animal shows (D) the newly implanted spermatophore from which sperm are beginning to move out, (E) a strong posteriorly directed stream moving along the lateral body wall immediately above the right anterior fin, (F) the arrival of the stream over the right gonopore where it begins to traverse the dorsal body wall towards the left gonopore until (G) accumulations of sperm build up over each gonopore. (H) The penetration of sperm through the left gonopore into the sperm pouch (which can be seen running anteriorly, exterior to the ovary containing eggs, by virtue of the sperm filling it.) Some sperm still remain outside of the body wall. (I) A spermatophore artificially attached on the dorsal body surface, which illustrates the migration of sperm streams to the lateral body wall on both sides rather than (as in *Spadella*) travelling along the dorsal body wall. (J) The remains of a spermatophore artificially attached immediately behind the head laterally on the collarette, streams from which have meandered in the region failing to establish a gonopore-oriented direction to their flow. Subsequent to this photograph, they became diffuse and disappeared. The approximate length of these mature animals is 9 mm and width of the trunk region 0.5 mm. Abbreviations are: fa—anterior fin; fp—posterior fin; g—gonopore; o—ovary; p—seminal pouch; s—sperm; sp—spermatophore; sv—seminal vesicle.

frequency and violence of the muscular movements of the partners is much more intense than in normal swimming movements. Attached head-on (Fig. 1 A–C), their bodies flex and twist in close proximity along their lengths as they move upwards in a spiralling motion followed by intervals of rest when they sink downwards.

At least 50 such encounters have been observed. Usually the encounter ended with both animals gaining and losing a spermatophore (Fig. 1 C). Only in situations where a single spermatophore was exchanged could its origin be in no doubt although we assume that all transferences were from the other animal. The moment of transference was never seen (the swimming movement appeared blurred when photographed with a flash of 1/250 sec duration). The site of attachment was always on the lateral trunk wall, often between the anterior and posterior fins, which is about one-third of the total body length anterior to the seminal vesicles. Although sometimes it varied in either direction, it was not to be found on the tail or head, or mid-dorsally or ventrally, nor on "the dorsal median line directly behind the ciliary loop" as in *Spadella* (Ghirardelli, 1968). No phenomenon has ever been seen in our populations in which animals were oriented head-to-tail, as occurs in *Spadella* while one is attached to a substrate, or as Murikami saw in *Sagitta crassa.* On one occasion three animals were observed attached at their head ends, and on separating at least two had lost and received spermatophores.

Immediately after receipt of a spermatophore the animal may be removed from the aquarium, placed in a small dish and viewed microscopically. Invariably, within the few seconds which elapse prior to examination, sperm are already streaming across the body surface (Fig. 1 D). Since no empty spermatophore is left behind, we believe that the "spermatophore" consists of the tightly packed sperms enveloped in a thin layer of material of presumably adhesive mucoid nature which disintegrates as the sperm stream through it. Ghirardelli (1968) refers to the phenomenon as the dissolving of the spermatophore. Although several streams radiate out in various directions at first (Fig. 1 E), only the stream which proceeds laterally in a posterior direction continues to move, and it does so until it reaches the female gonophore on that side (Fig. 1 F), which is situated close to the junction of the trunk and tail. Some of the sperm then travel around the circumference of the body to the gonopore on the opposite side, so that both pores have accumulations of sperm over them (Fig. 1 G). The sperm enter and migrate along the seminal receptacle or pouch which extends along the outer edge of the ovary (Fig. 1 H). The process by which internal fertilization of the eggs then occurs is described in detail by Ghirardelli (1968) who provided a series of photographs showing a pattern of sperm migration from the dorsal body wall behind the ciliary loop in *Spadella.* In this case, the sperm path bifurcates to reach both gonopores at the same time. In the series of photographs in Figure 1 D–G, the elapsed time from spermatophore attachment at the level of the anterior end of the anterior fin to entrance at the gonopores was 10 minutes. The rest of the sperm which had not reached the gonopores quickly lost motility and are presumably detached during swimming movements of the animal, because after a further 10 minutes there are none remaining on the outside of the body. This condition varies from *Spadella,* where nearly all the sperm travel to and enter the gonopores.

Self-fertilization

In the course of experimental work involving the measurement of mature live animals over a long period of time, five individuals were accidentally observed where sperm were emerging from a small rupture of the seminal vesicle anteriorly and migrating along the lateral edge of the tail, entering the gonopore on that side. Relative to the volume of sperm in the seminal vesicle, the sperm were very few in number. The animals subsequently died before laying eggs.

Self-insemination can be artificially induced by breaking open a full seminal vesicle using a very fine needle which causes the spermatophore to "pop" out. By confining the animal to a small dish in which the depth of water is only 2–4 mm, the spermatophore can usually be maneuvered to touch the body of the animal where it sometimes adheres. When placed on the lateral trunk wall, the sperm stream out according to the sequence of events described in the previous section. This procedure was performed only on animals which had been isolated before becoming mature, at a stage when seminal vesicles had not begun to develop, ovaries were tiny and contained only stage I eggs (both conditions which are known not to be reverted to after maturity in this species, Reeve, 1970b) and which were smaller than any mature individuals. This was to insure that there was no possibility that prior insemination could have taken place. A total of 41 animals which were inseminated by their own sperm in this manner laid eggs which developed into larvae. None laid infertile eggs. Most of the animals which did not survive to lay eggs died during or within hours of handling, many obviously damaged in the process.

In the preceding experiments, spermatophore adhesion was directed to the region where it occurs naturally in cross-fertilization. Some attempts were also made to place spermatophores on other parts of the body. Anterior to the ventral ganglion, including the region of the ciliary loop, sperm trails issued from the spermatophore in various directions but none travelled far back towards the gonopore (Fig. 1-J). Results were less clear when spermatophores were placed on the tail. No strong directional flows occurred, but the close proximity of the gonopore and the flicking of the tail fin during swimming attempts were both probably factors in permitting a few sperm to occasionally reach the gonopores. It was difficult to obtain adhesion of spermatophores dorsally or ventrally, and where this occurred, as in the dorso-lateral position of Figure 1-I, sperm trails would first orient themselves laterally often on both sides of the body before moving towards the gonopore. Unlike the condition in *Spadella,* therefore, sperm could not be induced to move down the mid-dorsal line of the body wall.

Relative frequency of self- and cross-fertilization

It was reported (Reeve, 1970b) that larvae could result from eggs laid by isolated individuals. Such occurrences are infrequent but since that study was concerned with estimates of fecundity based on egg-laying, quantitative data on fertility were not obtained. The maturation and laying of eggs in *Sagitta hispida* is not dependent on prior insemination and fertilization.

In a new series of experiments, 66 animals out of 86 isolated from immaturity as described above laid one or more batches of eggs amounting to a total of 186 batches. Only four batches (2%) resulted in the production of larvae which themselves amounted to less than 17% of the number of eggs laid in those four batches. No batches were discarded until beyond the normal period required for hatching (36–48 hr) although fertile eggs would cleave within 10 minutes of being laid.

Since it is possible that the confinement of a normally planktonic 9-mm long animal in 250 ml could adversely influence its ability to produce offspring, a preliminary experiment was run in which 10 pairs of immature animals were utilized. Only three pairs survived long enough to produce a batch of eggs, all of which were fertile. This experiment was not expanded because of mortality problems probably aggravated by cannibalism. The following experiment, however, demonstrated the fact that larvae could be produced in close confinement.

This experiment was performed primarily to ascertain whether an animal once inseminated could store sperm for fertilization of subsequent batches of eggs. This ability is possessed by planktonic marine copepods (*e.g.,* Marshall and Orr, 1955). Observations suggested that sperm storage was unlikely because there were no visible signs of sperm in the seminal pouches on the day following insemination (using ×12 magnification). Initially, their position can be seen even by the unaided eye.

Mature animals with well-developed ovaries were removed from aquarium populations and isolated. Of 32 animals isolated, 24 subsequently laid a batch of eggs, of which 10 were fertile and resulted in larvae only slightly fewer in number than eggs laid. A total of 23 further batches were laid by this group, all of which were infertile. In the case of those whose first batch was infertile, we presume that they had been removed from the population before insemination had occurred. There was no evidence that insemination conferred fertility on any but a single batch of eggs.

Discussion

On the basis of our own observations of *Sagitta hispida,* we would agree with Alvariño that cross-fertilization by copulation is the rule. Strictly, most observations in the literature have concerned insemination rather than fertilization. Our indication of successful fertilization was the hatching of a larva, but we have no information on the viability or ultimate fecundity of such larvae. In *Sagitta hispida* our observations suggest that larvae are normally produced following insemination during copulation which can sometimes be proved to be cross-insemination (where only a single spermatophore is involved). Self-insemination is rare but has been shown to occur, and can readily be artificially induced to produce fertile eggs.

It might be argued that transference of a spermatophore from the seminal vesicle to the trunk of the same individual might occur during copulatory activities, whether accidentally or not. The chances of the seminal vesicle coming into contact with another part of its own body would seem much lower than of touching the other animal. On one occasion only, a single mature individual in an aquarium was seen to be violently twisting its body as in the copulatory sequence. There was, however, no spermatophore relocation.

The obvious method by which two planktonic chaetognaths could become attached for copulation is by use of their grasping spines, since their other appendages, the fins, contain no musculature (Ghirardelli, 1968). We do not know whether an act of cannibalism is behaviorally different from copulation. When an individual seizes an animal smaller than itself, the wider spread of its spines enables it to encircle and render ineffective the spines of the smaller animal. When animals are of similar size, as two mature animals would be, it would be much more difficult for one to gain the advantage. In the ensuing struggle, spermatophores may be transferred and the animals may separate because neither can get a decisive grip on the other. In support of this possibility that copulation and attempted cannibalism are different results of the same process, we have occasionally seen mature animals in aquaria which have just ingested another chaetognath which appeared to be of similar size, where the attacker bore a spermatophore on its body. This strongly suggested that both copulation and cannibalism had occurred.

Reeve (1966) noted that he had never observed cannibalism in *Spadella cephaloptera* and this observation still holds true in observations of the breeding population which we maintain in this laboratory. He suggested that the benthic *Spadella* might have developed a mechanism to prevent self-predation based on recognition of different kinds of swimming vibrations, since it congregated on surfaces rather than being dispersed in a 3-dimensional space where there was less chance of encountering a member of the same species rather than a food organism such as a copepod. Since copulation in *Spadella* is achieved with the aid of adhesion of one of the pair to the substrate, the mechanisms of cannibalism are not necessary for copulation.

The distinction between *Spadella* and the planktonic chaetognaths is not absolute. *Sagitta hispida, S. helenae* and possibly other neritic species possess the ability to attach to surfaces such as the aquarium wall. Unlike *Spadella,* however, where the newly hatched larvae attach on hatching and 95% of the population is usually attached at any one time, *Sagitta hispida* does not attach significantly until approaching maturity and even then most of the population is usually to be found swimming in the water column, in aquaria. Egg masses on aquarium walls indicate that *S. hispida* utilizes surfaces for egg-laying, but we have not as yet seen indications that this species can transfer spermatophores while attached to a surface.

Ghirardelli (1968) was also able to induce self-insemination in *Spadella cephaloptera* although he had no evidence that it could occur unaided, and his observations of sperm migation in this region suggested that it was unlikely. Our observations suggest also that it is unlikely, but that it can occur. In *Sagitta setosa,* on the other hand, Dallot (1968) maintained isolated individuals in which 50% of the spawnings gave fertile eggs. He did not observe any phenomena indicative of cross-fertilization, although he did not discount its existence in that species.

Since *Sagitta hispida* is the only planktonic species currently being routinely raised over its whole life cycle in the laboratory, it is clearly too early to make generalizations covering the entire range of planktonic Chaetognatha with respect to relative frequency of cross- and self-fertilization. If, indeed, self-fertilization is a regular phenomenon which becomes more likely when opportunities for cross-fertilization are minimal, it might be expected to occur more frequently in species habitually in lower densities such as typically oceanic and bathypelagic ones.

This work was supported by NSF Contract No. GA 28522X. Our thanks are due Drs. Harding Owre and Mary Rice for their interest, advice and reading of the manuscript.

SUMMARY

1. The first description is provided of the act of copulation of a planktonic chaetognath which leads to successful insemination and fertilization. It consists of a behavioral sequence in which partners maintain contact at their head ends while engaging in violent movements which result in the transfer of spermatophores.

2. Acts of self-insemination in the same species are also described, as well as experiments in which self-insemination and successful fertilization are induced.

3. The consequences of attachment of spermatophores to various parts of the body are described and compared to observations on the benthic genus *Spadella*.

4. Experiments showed 2% of batches of eggs laid by individuals isolated prior to maturity produced hatchings, suggesting that self-fertilization, although uncommon, does occur naturally.

5. Experiments indicated that one insemination was effective for only a single batch of eggs.

6. The similarities between copulation in *Spadella* and *Sagitta* are discussed and the possibility that self-fertilization presents a short-term survival mechanism for populations at low density is suggested.

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Reference: *Biol. Bull.*, **143**: 215-221. (August, 1972)

EXCYSTATION OF THE APOSTOMATOUS CILIATE, *HYALOPHYSA CHATTONI,* WITHOUT METAMORPHOSIS [1]

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Apostome ciliates are found encysted on the exoskeletons of Crustacea, excysting at the molt of their host (Chatton and Lwoff, 1935). A major group, the exuviotrophs, excyst and feed only on the fluid from the host's molt; while another large group, the histotrophs, also excyst at the death of an injured host and feed on the released body fluids. In either case the excystation of the encysted form (phoront) is preceded by an extensive metamorphosis. In the histotrophs the metamorphosis occurs soon after the migratory form settles on the host, but exuviotrophs metamorphose immediately before the ecdysis of the host. The result of this metamorphosis is a feeding stage (trophont) capable of engorging up to 30 times its initial volume (Bradbury and Trager, 1967b). After this rapid feeding, the apostome encysts and divides into numerous small ciliates (tomites) which seek out and settle on a new host.

Previous studies (Miyashita, 1933; Chatton and Lwoff, 1935; Trager, 1957; Bradbury and Trager, 1967b) suggest that the exuviotrophic phoronts are stimulated to metamorphose and excyst by the leakage of some compound or mixture of compounds that builds up in the host prior to ecdysis. Accordingly, the phoront stage of *Hyalophysa chattoni* found on brackish water shrimps was subjected to solutions of various substances that were likely to be concentrated in the shrimp just before ecdysis.

MATERIALS AND METHODS

Grass shrimps (*Palaemonetes pugio* and *P. intermedius*) were collected near the Pamlico Sound Research Station, at Aurora, North Carolina. As many as several hundred were collected and held in two ten-gallon aquaria filled with 13.3‰ artificial sea water salts (Aquarium Systems Inc.), and equipped with aerater and filter. While the water temperature (22 to 25° C) and specific gravity (1.015) remained fairly constant, the pH varied between 6 and 8. The shrimps were fed daily. Of the 298 molting cycles recorded, 70% were 1 to 3 weeks long. All of the fresh molts contained *Hyalophysa chattoni* [identified by silver impregnation by the Chatton-Lwoff (Corliss, 1953) and the Protargol (Kirby, 1950) procedures].

Premolt shrimps were recognized by the gap that widens between the newly formed exoskeleton and the ends of the antennal scales and uropods as ecdysis nears (Passano, 1960). Metamorphosis in living *Hyalophysa* was recognized by crowding of food plaquettes to one side and the mid-ventral position of the contractile vacuole (Bradbury and Trager, 1967a).

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[1] This work is part of a thesis submitted to North Carolina State University, Raleigh, in partial fulfillment of the requirements for the M.S. degree in Zoology.

Phoronts for *in vitro* experiments were obtained by cutting off a patch of exoskeleton containing numerous phoronts. On the shrimp, *Hyalophysa* preferentially settles in the cuticular depression at the base of the eyestalk. The exoskeleton of the living shrimp was cut from the base of the rostrum, medial to the base of the eyestalk beyond the field of phoronts. The second cut from the ventral edge of the carapace extended dorsally to intersect the first cut. The final cut was made parallel to the body surface just under the exoskeleton severing the musculature and connective tissue. These three incisions released a fragment that included the anterior end of the gill chamber, the antennule, the antenna—including the antennal scale, the eyestalk, and the base of the rostrum.

The fragment was washed vigorously in brackish water (13.3‰ artificial sea water with 500 units penicillin/ml and 0.05 mg streptomycin/ml (Bradbury and Trager, 1967b). The underlying muscle and connective tissue were picked away with forceps leaving only pieces of exoskeleton with the attached phoronts. During this half-hour procedure, the pieces were washed at frequent intervals to dilute the released body fluids.

All experimental solutions were made from the antibiotic brackish-water solution. All controls were in the antibiotic brackish water alone.

The cleaned pieces of exoskeleton were placed in 1 ml samples of solution in clear plastic disposable depression dishes (Scientific Products) and the entire dish was covered with "Parafilm" (American Can Company). A typical experiment would include 4 replicates of the control and 4 replicates of the experimental solution using phoronts from the same shrimp. The number of phoronts in each depression was counted and the percent of excystation was calculated by counting the remaining cysts at 4 hour intervals for 24 hours.

To obtain body fluid from uninfected *Palaemonetes*, the shrimp was crushed in about 0.5 ml of the antibiotic brackish-water solution. The fluid was collected in a hypodermic syringe and mixed with equal parts of the antibiotic brackish water.

All chemicals, except the ecdysterone, used to make experimental solutions were obtained from Sigma Chemical Company. The ecdysterone came from Mann Research Laboratories.

RESULTS

A surprising observation in the course of these experiments was that the excysting phoronts on intermolt shrimps resemble in all visible respects the normal migratory stage, the tomite (Bradbury, 1966). No compound used in these experiments triggered metamorphosis of phoronts on intermolt shrimps. The phoronts were tested for excystation from 2 to 12 days after settling on the shrimps. In all experimental solutions, including 0.05 M Tris-HCl buffer (pH 9.0), 0.5% β-D glucose, 0.5% glycogen (from shellfish), 0.5% N-acetylglucosamine, and 10^{-7} M ecdysterone, the immerging ciliate had the body form and ciliary pattern of the tomite.

While the phoronts on intermolt shrimps always excysted as tomites, those on permolt shrimps excysted either as tomites or trophonts. In premolt shrimps

Table I

Averaged per cent excystation of trophonts and tomites from replicate experiments using 4 individual premolt shrimps*

Solutions	# Cysts	Hours					
		4	8	12	16	20	24
Control	156	51%	58%	83%	87%	87%	87%
I Glycogen (0.5)%	309	45	75	90	96	96	96
Control	234	21	69	90	90	90	90
II Glycogen (0.5%)	200	5	82	95	96	96	96
Control	111	13	39	97	99	99	99
III Glucose (0.5%)	182	12	22	98	100	100	100
Ecdysterone (10^{-7} M)	118	8	23	97	100	100	100
Control	48	45	61	82	87	87	87
IV Glucose (0.5%)	71	55	77	92	97	97	97
Glucosamine (0.5%)	88	55	69	85	91	93	93

* Per cent excystation was calculated after counting the remaining cysts. Only trophonts were found swimming in the dishes after 4 hours. Both trophonts and tomites were found after 8 hours. After 12 hours only tomites were found.

(Table I) phoronts excysted as trophonts within 4 hours and continued to excyst as trophonts for another 4 hours. But after 8 hours, the remaining phoronts excysted as tomites. Excystation of tomites from intermolt shrimps (Table II) started after an initial lag of 4 hours and increased rapidly to 80% or higher by 12 hours. Excystation was slower from 12 to 16 hours with little occurring after 16 hours. The general pattern of per cent excystation for both intermolt and premolt shrimps was the same after early differences. No significant effects on per cent excystation due to treatments alone were observed in any of the experiments. Transformation and analysis of variance of data for each shrimp were carried out using the Statistical Analysis System developed at NCSU for the use of the IBM 360/75 computer at Triangle Universities Computing Center.

In light of these unexpected results and because experiments using blood have resulted in excystation of trophonts from other crustaceae (Miyashita, 1933; Chatton and Lwoff, 1935; Trager, 1957; Bradbury and Trager, 1967b), shrimp body fluid was used as an experimental solution, with intermolt shrimps.

Experiment 1

Three live infested shrimps were placed in body fluid from other shrimps diluted 1:1 with antibiotic brackish water. No excystation was observed after 24 hours.

Experiment 2

Two fields of phoronts were cut from a shrimp 4 days from its last ecdysis. Neither piece was cleaned. One was placed in extra body fluids, while the other was placed in the antibiotic brackish water alone. Excystation of tomites occurred from the first piece after 2 hours and from the second after 9 hours.

Experiment 3

One shrimp was found to have phoronts on the bristles of its maxillae. These phoronts excysted much more slowly than phoronts from the cuticular depression at the base of the eyestalk of the same shrimp. At 8 hours, 90% had excysted as tomites in the depression while none on the bristles excysted until after 8 hours (73% at 12 hours).

Experiment 4

When shrimps killed by stabbing near the field of phoronts were left in brackish water, no excystation of the phoronts occurred. Apparently they died on their hosts.

To test whether the excysted tomites would behave as normal tomites, they were offered uninfested shrimps as hosts. The tomites darted about over the shrimp's body as normal tomites would, but none of the excysted tomites were observed to re-encyst.

TABLE II

Averaged per cent excystation of tomites from replicate experiments using 6 individual intermolt shrimps

	Solutions	# Cysts	Hours					
			4	8	12	16	20	24
	Control	50	0	43%	81%	83%	88%	92%
I	Glycogen (0.5%)	55	0	57	95	100	100	100
	Control	96	0	94	100	100	100	100
II	Glucosamine (0.5%)	108	0	87	96	98	98	98
	Control	61	0	90	97	97	97	97
III	Glycogen (0.5%)	33	0	63	97	97	97	97
	pH 9.0 (0.05 M Tris-HCl buffer)	28	0	100	100	100	100	100
	Control	26	0	29	58	69	74	84
IV	Glycogen (0.5%)	20	0	25	50	90	90	90
	pH 9.0 (0.05 M Tris-HCl buffer)	32	0	38	66	82	82	86
	Control	120	0	61	85	89	91	91
V	Glycogen (0.5%)	76	0	65	86	87	92	92
	pH 9.0 (0.05 M Tris-HCl buffer)	71	0	79	87	88	88	94
	Control	121	0	16	83	88	91	91
VI	pH 9.0 (0.05 M Tris-HCl buffer)	79	0	11	63	79	82	82

Discussion

Because glycogen, glucose, and glucosamine markedly increase in Crustacea prior to ecdysis (Passano, 1960; Florkin, 1960), these compounds seemed likely to leak from the host just prior to the molt and perhaps thereby induce morphogenesis in the exuviotrophic apostome. Glucose and gycogen were also tested in antibotic brackish water buffered at pH 9.0, the pH of molting fluid (Dennell, 1960). None of these substances effected metamorphosis of *Hyalophysa* on shrimp although the same species on the hermit crab has been reported to metamorphose in weak solutions of glycogen (Bradbury and Trager, 1967b). On the hermit crab, phoronts are found primarly between gill lamellae within the gill chamber (Bradbury, 1966). The phoronts found on hermit crabs never have been observed to excyst as tomites. In view of this as well as the results of Cleveland and Nutting (1955) with the flagellates of the wood roach, Trager (1957) has suggested that molting hormone might effect the metamorphosis of exuviotrophs. Accordingly ecdysterone was tested for its effect on excystation with no observable results. Perhaps, as Trager has suggested, a series of events must occur to induce metamorphosis and excystation.

Two variables in all the experiments could not be completely controlled: (1) the amounts of body fluid in contact with phoronts during dissection, and (2) the amount of tactile stimulation the cysts received. Although the pieces of exoskeleton were washed repeatedly with clean solutions during the cleaning of the pieces, the phoronts still were bathed in body fluids for as long as one half hour. Perhaps Experiment #3 with phoronts encysted on bristles was the only experiment in which these two factors might not be significant. Although the phoronts on the bristles excysted after a longer lag period and did not excyst in as great numbers, excystation still occurred.

It should also be considered that all the conditions of habitat provided by the shrimp were not duplicated *in vitro*. Living shrimps always have some part of their anterior body in constant motion. Antennae and antennal scales move, the scaphognathite beats continuously, the maxillipeds and mouth parts are usually in motion. These movements are in addition to the usual walking and swimming movements of the shrimp. The effect of such movements is to bathe the phoronts in a constant through variable stream of water. It would seem that such a constant flow of water would provide the phoronts with a good supply of oxygen. Since phoronts of all apostome species whose life cycle is known settle on well aerated sections of the exoskeleton, the phoronts may have a relatively high oxygen requirement. They do move within the cyst and their contractile vacuoles are active. None of these experiments tested the effect of the experimental substances moving over the surface of the body (*i.e.*, phoronts bathed in a moving stream of a substance for a time before the current was stopped).

Since phoronts die *in situ* on intermolt shrimps (dead from any cause), the loss of currents of water in addition to failure to release body fluids until decomposition begins may explain why these phoronts do not excyst. Another possibility is that the lack of oxygen affects the phoronts before the body fluids leak from the shrimp.

In Experiment #1 (living shrimps in 1:1 brackish water and body fluids) no excystation occurred even though the two conditions of water currents and body fluid were met. However, this experimental solution was cloudy and gummy with coagulated shrimp blood. Although antibiotics were added, bacterial action was just retarded—not stopped. The viscous water currents were unlikely to be comparable to water currents in the shrimp's natural environment. On the other hand, excystation may require both body fluids and cessation of movement. If this were so, the results of Experiments 2 and 3 would be expected because the large amount of body fluids in combination with a lack of water currents would lead to excystation. In Experiment #4 the stabbed shrimp would release body fluids, but they would be quickly diluted. The wound was behind the field of phoronts. This would mean that the flow of water would carry the body fluids away from the phoronts. The swift coagulation of shrimp blood would quickly stop any great loss of fluid. Thus this experiment would have to be considered inconclusive.

Since it was not feasible to give the cysts close microscopic scrutiny before removing them from the shrimp, it could not be ascertained whether metamorphosis of some had already occurred. Some premolt shrimps were taken very close to the oncoming molt. In these cases trophonts excysted during the dissections. It seems quite likely that metamorphosis had occurred before dissection was begun in such cases. It is also reasonable that the events leading to metamorphosis had already started for the other phoronts that excysted as trophonts. This excystation of trophonts is common when dealing with premolt shrimps. The unexpected result was the reappearance of the tomites.

However this reappearance of tomites does provide the evidence hitherto lacking for the independence of metamorphosis and excystation. Chatton and Lwoff (1935) imply from their discussions on metamorphosis of *Synophrya* that metamorphosis and excystation are in fact two different processes. Phoronts of apostomes have never been shown to excyst as tomites at any time before. Therefore, the readiness of the phoront to excyst under stress, and the reappearance of the tomite from the phoretic cyst were completely unexpected.

Even though the excysted tomites were not observed to settle on new hosts, their behavior was like that of tomites seeking a host in the normal course of their life cycle. Probably the tomite has only enough reserves to settle and encyst one time. Reserves remaining in the phoront are probably used for excystation. Since the tomite can not feed, its reappearance in this form is fatal to it.

In the course of this work no phoronts have been seen on the molt of the shrimp. Nor were empty cysts observed on intermolt shrimps. Apparently the normal life cycle proceeds without accident unless experimentally altered. But the results of this series of experiments establish that the ability to excyst is maintained throughout the phoretic stage.

I want to thank Dr. P. C. Bradbury the chairman of my graduate committee, and Drs. D. E. Smith and D. Huisingh for their guidance and help. I am grateful to Dr. Larry Nelson and Mr. Frank Verlinden for their advice and assistance with the statistical analyses of my experiments. This work was supported in part by a grant from the National Institutes of Health (RR-00011-09) for Computer Use in Health Sciences.

SUMMARY

Pieces of exoskeleton bearing *Hyalophysa* phoronts from the bases of the eye-

stalks of intermolt and premolt *Palaemonetes* were placed in antibiotic brackish-water solution containing 0.05 M Tris-HCl buffer at pH 9.0, 0.5% glycogen, 0.5% β-D glucose, 0.5% N-acetylglucosamine, and 10^{-7} M ecdysterone. Controls were placed in antibiotic brackish water alone. In all experiments, the phoronts showed the same rates of excystation in control and experimental solutions. The phoronts excysted as tomites from intermolt shrimps, while both trophonts and tomites excysted from premolt shrimps. Experiments using body fluids from the host shrimp have indicated that the substance causing this unexpected excystation of tomites is in the body fluids of the host.

The resulting excystation of tomites from the phoretic cysts of *Hyalophysa* establishes the separation of metamorphosis and excystation in apostome ciliates.

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LOSS OF LIMBS AS A STIMULUS TO ECDYSIS IN BRACHYURA (TRUE CRABS)[1]

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The common means of inducing precocious molts in Crustacea is by eyestalk extirpation (Zeleny, 1905), which removes the X-organ-sinus gland complex containing the postulated molt inhibitory hormone (MIH; Passano, 1960) and presumably allows the action of ecdysterone to initiate molt preparations. This procedure, though effective in stimulating molting in many species, frequently kills the animals either at the time of surgery or at the time of ecdysis itself. Such is the case with one of the experimental animals used in our laboratory, the land crab, *Gecarcinus lateralis*. Moreover, the injection of ecdysterone into unoperated animals frequently leads to the death of the animals even in species where it is an effective stimulus to molting (Krishnakumaran and Schneiderman, 1968; Williams, 1968; Skinner and Graham, 1970). Previously we reported that in *Gecarcinus* the loss of a large number of limbs, either pereiopods (walking legs) or chelipeds (claws), triggered precocious but apparently physiologically normal molts with high survival, whereas injections of ecdysterone were ineffective (Skinner and Graham, 1970).

It is known that regeneration of lost limbs occurs in the premolt period of the land crab (Bliss, 1956; Hodge, 1956a, 1956b; Skinner, 1958, 1962; Skinner and Graham, 1970) and many other Crustacea (Emmel, 1910; Bliss, 1960; Needham, 1965; Hay, 1966; Goss, 1969). Furthermore, the loss of numerous limbs triggers precocious molts in certain insects (Cameron, 1927) and the land crab (Bliss, 1956; Skinner and Graham, 1970). On the other hand, in some cases such as the cockroach, *Blatella*, loss of limbs before a certain critical time can as much as double the interval before the next ecdysis (O'Farrell, Stock and Morgan, 1956).

This paper represents a study of the interrelationships between molting and limb regeneration in the Brachyura, or true crabs. It includes observations on: (1) the effect of limb loss on the duration of the molt cycle of several species of marine Crustacea, with data on precocious molts caused by eyestalk removal included for comparison; (2) the minimal number of legs which must be removed to cause molting; (3) the effect of the total number of legs removed on the size of the subsequent limbs regenerated (regenerates); (4) the effect of lack of privacy and of light regimen on the duration of the molt cycle (Bliss and Boyer, 1964);

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[1] Research supported by the U. S. Atomic Energy Commission under contract with Union Carbide Corporation.

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(5) the interaction between loss of regenerating limb buds in the early premolt period, duration of the premolt period, and re-regeneration of the lost regenerates. Experiments in (2), (3), (4) and (5) used the land crab, *Gecarcinus lateralis,* as the experimental animal.

MATERIALS AND METHODS

Specimens of the land crab, *Gecarcinus lateralis,* were maintained in the usual manner (Skinner, 1962). Specimens of two portunid crabs, (the blue crab, *Callinectes sapidus,* and the green crab, *Carcinus maenas*) the fiddler crabs, *Uca pugnax* and *U. pugilator,* and the spider crab, *Libinia emarginata,* were kept in sea tables with running water at the Marine Biological Laboratories, Woods Hole, Massachusetts. They were fed mussels.

Animals were caused to autotomize limbs (Wood and Wood, 1932) by cutting at the merus. Eyestalks were removed by cutting at the articulating membrane. To increase survival, one eye was removed on day 1, the other on day 2 or later.

A single leg was removed from control or eyestalkless specimens to permit us to detect an approaching molt by the progress of limb regeneration (Bliss, 1956; Skinner, 1962, 1965). Experiments on *Gecarcinus* were performed throughout the year; experiments on the marine crabs were performed in mid-June through August.

The size of limbs was determined by measuring the external dimensions of the various segments as well as by weighing the limbs. Postmolt limb weights were determined one month after ecdysis, since the limb immediately following ecdysis contains only some 17% of the final intermolt quantity of tissue isolable one month later (Skinner, 1966a) after a period of rapid protein synthesis has occurred (Skinner, 1966b).

RESULTS

Response of marine Crustacea to limb loss

Libinia: Neither controls (20), eyestalkless (8) nor animals with 6 or 8 legs missing (L. A. = legs autotomized animals; 20, total) molted or showed any sign of an approaching molt. Scabs remained on the autotomy plane throughout the experiment, which was terminated after 10 weeks (Table I).

Carcinus: Four animals missing 6 limbs regenerated legs and molted successfully; two were killed by other animals. No other animals (*i.e.,* eyestalkless or controls) showed any indications of an approaching molt. Eyestalkless specimens did show copulatory behavior typical of premolt animals, with the males grasping the females beneath them.

Callinectes: Both eyestalkless animals and those missing 6 limbs prepared for ecdysis with attendant limb regeneration. Of 10 eyestalkless specimens, all survived for several weeks after eyestalk removal, 3 survived until the late premolt period but only one until ecdysis (42 days after eyestalk loss). That animal died after having split the epimeral suture in the branchiostegite region and initiating emergence from the old exoskeleton. No appendages were freed at the time of death. The mucilaginous layer (molting fluid) was apparent. In the land crab,

TABLE I

Effect of autotomy of legs or eyestalk removal on the interval to ecdysis of marine crabs

Animal	Treatment	Effect	Days to ecdysis
Libinia (Spider crab)	E/S* (8)†	0	—
	6 to 8 L. A.‡	0	—
Carcinus (Green crab)	E/S (10)	0	—
	6 or 8 L. A. (6)	+	48 ± 5 (S.D.)
Uca pugnax (Fiddler crab)	E/S (24)	+	22 ± 3
	6 or 8 L. A. (24)	+	25 ± 2
Callinectes (Edible blue crab)	E/S (10)	+	42
	2 C. A. 4 L. A. (5)	+	51, 51, 61, 64, 70

* E/S = Eyestalks removed.
† ( ) = Number of animals in sample.
‡ L. A. = Legs autotomized.

Gecarcinus, eyestalkless animals commonly die slightly later, when they have freed themselves almost completely from the old exoskeleton.

The interval to ecdysis was longer in animals triggered to undergo precocious molts by limb loss than in those stimulated by eyestalk extirpation which removes the MIH (Table I). However, the viability of the L. A. animals was considerably greater. In addition to 5 animals that molted, two other groups of 6 and 10 L. A. animals had responded, as witnessed by extensive limb regeneration. Unfortunately, that experiment had to be terminated before the animals underwent ecdyses.

Uca: Both *U. pugnax* and *U. pugilator* responded to both treatments. As in the case of *Callinectes,* there was a more rapid response to eyestalk removal, with ecdysis occurring within 20 to 25 days. The loss of 6 or 8 walking legs also stimulated premolt preparations, including limb regeneration. Ecdysis occurred approximately 25 days after limb loss. The mortality of both groups of experimental animals was high, with only one eyestalkless animal surviving ecdysis. Others also report high mortality of eyestalkless *Uca* (Fingerman and Yamamoto, 1967). However, the positive response of the animals to limb loss was clear (See Fig. 1, which shows photographs of *Uca* and other crabs stimulated to molt by limb removal.) As with *Gecarcinus,* removal of one appendage of the marine crustaceans could be useful as an early indication of an approaching molt (Bliss, 1956; Skinner, 1962).

Effect of eyestalk removal as compared to limb loss on precocious molts

We and others (Passano, 1960; Skinner, 1968) have for years used eyestalk extirpation as a means of propelling various species of Crustacea into precocious molts. The lethargic postoperative behavior of the animals, as well as their demise at the time of the subsequent ecdysis, indicated that although their macromolecular metabolism appeared indistinguishable from that of normal premolt animals (Skinner, 1965, 1966a, 1966b), nonetheless their premolt period was ab-

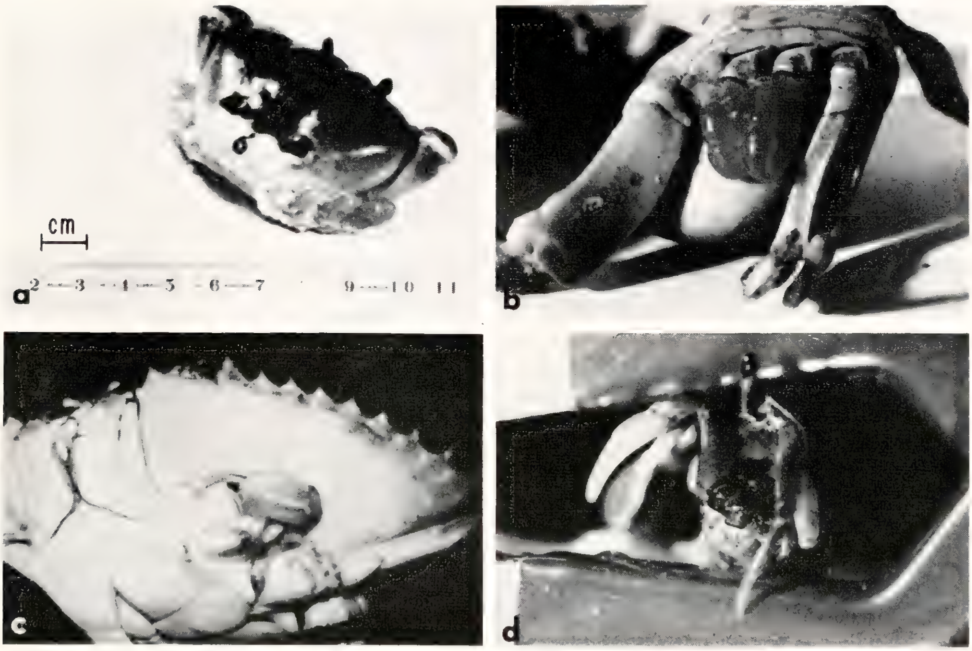

FIGURE 1. Specimens of crabs with regenerating limb buds. (a, b) *Gecarcinus lateralis*, (c) *Callinectes sapidus* and (d) *Uca pugnax*. Eight, 4, 6, and 6 walking legs were removed from animals (a, b) 45, (c) 36, and (d) 20 days previous to the time of photography.

normal. We have performed a series of experiments on *Gecarcinus* to compare the relative effectiveness of eyestalk extirpation and limb loss in inducing precocious molts.

We find that eyestalk removal leads to the most rapid molting preparations (Table I) but is accompanied with 100% mortality at the time of ecdyses (Fig. 2). Molts induced by limb loss, though somewhat delayed in time, almost always lead to healthy, normal animals (Fig. 2). Even those specimens deprived of 8 walking legs, though clumsy in their movements, molt successfully almost 100% of the time. Indeed, in most experiments, we observe no deaths following such treatment.

Minimum number of limb autotomies required to stimulate precocious molting preparations

These and the remaining experiments in this paper were performed with *Gecarcinus*.

Loss of from 1 to 4 limbs had no effect on the duration of the molt cycle (Table II). Animals which have lost 5 limbs, on the other hand, respond except when kept in community tanks. Loss of from 6 to 8 limbs, including various combinations of chelipeds and pereiopods, is almost 100% effective. Since in *Gecarcinus* the chelipeds comprise as much as 35% (21 to 35%; Table III) of the total body weight, whereas the walking legs contribute 11 to 17% (Table III),

TABLE II

Effect of autotomy of legs on interval to ecdysis in Gecarcinus. *Abbreviations are: L. A. = walking leg autotomized; C. A. = cheliped autotomized; (a) = In jar, ca. 10 min light/week; (b) = In jar, ca. 10 hr light/week; (c) = Community tank (6–8 animals); 10 hr light/week*

Treatment	Animals (No.)	Mean time to ecdysis (Days ± S.D.)
1 L. A. (a)	14	>200
(c) stock supply	>300	>200
2 C. A. (a)	6	>200
4 L. A. (a)	6	>165
5 L. A. (a)	7	86 ± 17
(b)	8	86 ± 8
(c)	8	>131
2 C., 4 L. A. (a)	8	77 ± 25
6 L. A. (a)	8	89 ± 18
8 L. A. (a)	8	70 ± 12
(b)	7	81 ± 17

loss of two chelipeds should have been effective in stimulating molting if the stimulus were loss of tissues mass. However, such was not the case.

The duration of the premolt period is dependent on the size of animals, being

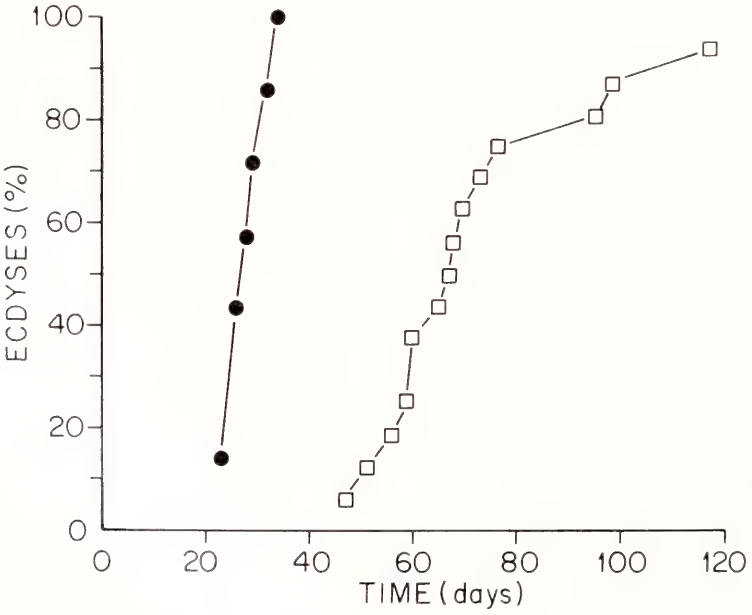

FIGURE 2. Effect of eyestalk removal or autotomy of limbs on the duration of the intermolt period of *Gecarcinus*. Eyestalks were removed on successive days from 7 animals ● — ●, all of which died at ecdysis; 8 walking legs were autotomized from 15 animals □—□. The animals were housed in individual bottles and observed until ecdysis occurred.

Table III

Per cent body weight comprised by claws or walking legs of Gecarcinus lateralis. *Animal 3 was female; all others were males*

	Total weight (Grams)	% Body weight	
		2 Chelipeds	8 Pereiopods
Animal 1	53.0	22	17
2	60.4	22	15
3	63	21	11
4	64.5	27	14
5	67	31	16
6	96	35	13
7	99	32	17

longer for large animals than for small. For example, animals of carapace width 4.2 to 4.7 cm underwent ecdyses 33 to 54 days after limb autotomy, whereas animals of carapace width 5.2 to 5.3 cm took 50 to 82 days. Therefore, in any one experiment, animals of similar size were used.

Environmental effects on the length of the molting cycle

When crabs lacking either 6 or 8 walking legs are maintained in bottles, which are stored in relatively light-tight cabinets, they receive approximately 10 min of light per week. Such animals molt sooner than those maintained in community tanks that receive 10 to 12 hours of light per week during the routine cleaning and feeding operations (Table II). This observation confirms a previous report of Bliss and Boyer (1964). The following experiment demonstrated that the effect was due to privacy rather than the amount of exposure to darkness. A series of animals was maintained in bottles kept outside the cabinets usually used for storage of experimental animals. Thus these animals received the same exposure to light as did the animals kept in groups of 5 or 6 in community tanks. These animals molted as soon as did animals kept in bottles in the dark (Table II).

It should be noted that the marine crabs studied were maintained in community tanks. Since limb removal stimulated precocious molts in all species of marine animals (*Callinectes, Cancer, Uca*) except *Libinia,* it may be that *Libinia* is more sensitive to the presence of other animals than the other marine crabs.

Effect of total number of limbs removed on the size of the regenerate

Regenerated limbs are somewhat smaller than their non-regenerated counterparts. Quantitation of the size difference leads to the following observations: (Table IV) Compared to those of bilaterally symmetrical partners, the weights of non-regenerated limbs taken from 29 animals varied by no more than 4%, whereas the weights of regenerated limbs were smaller by approximately $\frac{1}{3}$ when from 1 to 6 limbs were removed. Since there are no normal, bilaterally symmetrical limbs left on crabs that have been induced to regenerate 8 walking legs, the weight

Table IV

Effect of the removal of one to eight limbs on the size of regenerated limb. Regenerative load refers to the number of limbs regenerated during a molting period. One or more months after ecdysis, limbs were removed and weighed. Regenerated legs were compared to bilaterally symmetrical non-regenerated legs. To test individual variability, crabs were induced to autotomize a pair of normal limbs. The data on 8 L. A. animals were obtained by comparing the weights of legs of normal animals of the same size (see text)

Regenerative load (walking legs)	Percentage change in weight between any two walking legs	Sample size
0; (individual variability)	± 4.4	29
1	−34.5	6
6	−34.7	16
8	−48.4	9

of such legs was compared to the weight of non-regenerated (normal) legs of a series of crabs of the same carapace width, a fixed parameter useful for comparing animals of different stages of the molt cycle. Loss of 8 walking legs leads to a striking decrease in the mass of tissue contained in each limb (Table IV). Rough calculations of the mass of tissue regenerated indicated that an animal (of the sizes used in these experiments) was capable of synthesizing approximately 4 grams of tissue which was distributed among the regenerated appendages. Thus, if 6 limbs were regenerated, each weighed $\frac{1}{3}$ less than non-regenerated partners whereas if 8 were regenerated, each weighed $\frac{1}{2}$ that of its non-regenerated control.

Effect of removal of partially regenerated limbs on the time of ecdysis

In another series of experiments (Skinner and Beattie, 1971), we have been studying the metabolic and biosynthetic properties of regenerating limbs. For those experiments, we cause the animals to autotomize regenerating limbs at various stages in their development. In one of our early experiments, limb buds of the same external dimensions and appearance were removed from 3 crabs. Two of the crabs molted within 10 days (see Animals 6 and 7, Fig. 3), without regenerating the limbs removed. The third crab, which molted 35 days after the removal of its limb buds, had regenerated those removed (Animal 19, Fig. 3). Apparently, there was a delay in the time of ecdysis sufficient to permit reformation of the limbs.

Since the length of time from one ecdysis to the next is not clearly defined in the land crab as it is for ecdyses specific to particular insect larval instars, it is not possible to determine the magnitude of the retardation in time of ecdysis with certainty. However, in observations on 30 animals (Fig. 3), no animal which underwent ecdysis within 25 days after loss of partially regenerated limbs had replaced those removed. By contrast, all animals which molted 33 or more days after autotomy of limb buds had re-regenerated. Thus the shortest time required for re-regeneration was 33 days after autotomy of partially regenerated limb buds. This corresponds favorably with the time required from eyestalk removal to ecdysis,

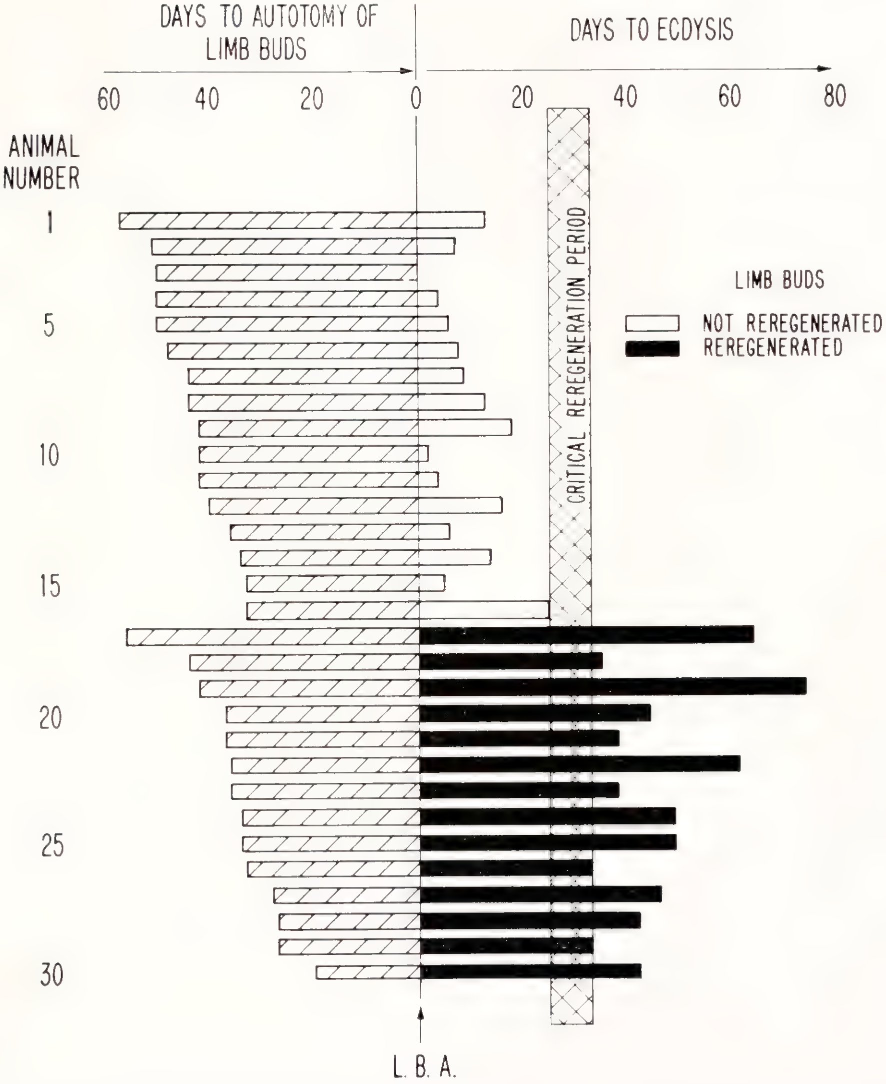

FIGURE 3. Effect of removal of regenerating limb buds on the duration of the premolt period of *Gecarcinus*. Molting preparations were initiated in 30 animals by limb removal and at some later date (Day 0 in this figure) regenerating limb buds were autotomized (L. B. A.). Animals were observed to the next ecdysis. The total length of the horizontal bar represents the duration of the complete premolt period. At the time of the next ecdysis it was noted whether the limbs had been re-regenerated. For purposes of graphical presentation here the animals are ordered into 2 groups: (1) numbers 1 through 16 are those that did not re-regenerate; (2) 17 through 30 are those that did. Within each group, the animals are listed from the longest to the shortest interval between initiation of molt preparations and limb bud autotomy. Note that the duration of the premolt period in animals that did not re-regenerate (Group 1) varied from 38 to 70 days, while in those that did re-regenerate (Group 2) the premolt period varied from 60 to 120 days. The cross hatched bar indicates the critical period (see text).

with attendant limb regeneration (Fig. 2), which was 27 days in animals of similar size.

Discussion

We have now demonstrated that the loss of a large number of limbs causes precocious molts not only in the land crab but also in three species of marine crabs. Two of these also prepare for ecdysis soon after eyestalk removal (*Callinectes* and *Uca*) but frequently die at the time of or shortly after ecdysis. The third (*Carcinus*) does not prepare for molting after eyestalk removal if greater than a certain size (approximately 2 cm carapace width, Bauchau, 1961; these data). Our experiments indicate that in large specimens of the green crab, limb loss is a more effective stimulus to ecdysis than is eyestalk removal.

Our experiments on the marine crabs, though preliminary, show that precocious molts triggered by loss of a critical number of limbs lead to apparently normal, healthy ecdyses, which the animals survive with as high viability as from normal ecdyses. Thus, this appears to be the method of choice for inducing precocious molts.

We do not suggest that these experiments demonstrate the presence of a molt-inhibitory factor in the limbs of Crustacea. A more likely hypothesis is that the stimulus to precocious molts is due to the severing of a critical number of nerves.

It would be of interest to attempt to stimulate precocious molts by severing the nerves to the walking legs of crabs. We expect that these experiments would prove to be technically difficult because of the rapidity with which the animals, at least *Gecarcinus,* autotomize limbs when confronted with noxious stimuli, such as anesthetization by exposure to the cold (4° C) or the injection of materials into the base of the appendage.

Cameron (1927) found that the interval between molts in another arthropod, the centipede *Scutigera forca,* was shortened by one half the normal time when *all* its legs were removed. Loss of as many as 26 of the 30 legs was ineffective in shortening the duration of the molting cycle. Unlike *Scutigera,* loss of *all* limbs apparently inhibits molting preparations in *Gecarcinus.* Two of our specimens of *Gecarcinus* lost all of their limbs in combat with other crabs. Both had normal eye reflexes and bore no evidence of other bodily injury. Yet, there were no signs of regeneration after 3 months, although other animals from the same shipment missing 6 or 8 walking legs had long since regenerated their lost limbs and undergone ecdyses. Since specimens of *Gecarcinus* respond within 3 weeks by basal growth of autotomized appendages, and since the limbless animals were hand-fed, it is unlikely that starvation was the explanation for their lack of response, especially since Cameron (1927) found that starved specimens of *Scutigera* survived one ecdysis and lived well into the next intermolt period (approximately 60 days).

A phenomenon similar to the critical period for re-regeneration of limbs in *Gecarcinus* (Fig. 3) was reported in the insect, *Blatella* (O'Farrell and Stock, 1953). Under conditions when the duration of the first instar was 12 days (25° C), loss of one limb between days 1 and 4 increased the period until the next ecdysis by 3 days. The total duration of the instar was then 15 instead of 12 days. At higher temperatures (30° C), the intermolt duration was increased from 5 to

6 days by the loss of one limb. A limb lost after a cretain critical period was not regenerated by the time of the next ecdysis. Rather, it remained as a papilla, to be regenerated during the next premolt period (O'Farrell and Stock, 1953).

The crustacean premolt period may be subdivided into 5 parts, designated stages D_0 through D_4 (Drach, 1939; Skinner, 1962, 1966a). A substantial fraction, if not all, of limb regeneration occurs in D_0 (Skinner, 1962). It has long been known that there is a critical time in the arthropod premolt period (O'Farrell *et al.*, 1956; problably D_0 in crustaceans; Skinner, 1962, 1966a) before which limbs must be removed if they are to be regenerated by the first ecdysis following limb loss. The ability of an animal to prolong D_0, in which it synthesizes another set of limbs and concomitantly delays the next series of premolt events (such as apolysis, the separation of the epidermis from the old exoskeleton, Jenkin, 1970; resorption of the old exoskeleton and synthesis of a new exoskeleton, Skinner, 1962) points up the complexity of the controls over various premolt phenomena. Our observations indicate that even if a single "on" switch is thrown to initiate the premolt period, the ordered series of events leading to a normal ecdysis do not proceed on an invariant time schedule. The whole premolt process appears to be self-monitored in such a way that subsequent events cannot proceed until early events reach a critical stage of completion and the *duration* of the critical period can be greatly extended. In fact, we think that the animals that did not re-regenerate were already in stage D_1 (and that the epidermis had already separated from the old exoskeleton when their regenerating limb buds were autotomized). Clearly, there is no turning back. The interacting controls are such that the animals did not even initiate the re-regeneration process.

We wish to thank W. G. Beattie and E. Ang for their help with some of these experiments. Some experiments were performed by the senior author in conjunction with the Experimental Invertebrate Course at the Marine Biological Laboratory, Woods Hole, Massachusetts. The help of the collecting staff and students in the course was greatly appreciated, as were the photographs of *Callinectes* and *Uca*, taken by Mr. P. J. Oldham of the Systematics-Ecology Program. D. E. Graham was an Oak Ridge Graduate Fellow under appointment from the Oak Ridge Associated Universities.

SUMMARY

1. Loss of 6 to 8 pereiopods or chelipeds triggers precocious molts in a number of marine crabs including the green crab, *Carcinus maenas,* the blue crab, *Callinectes sapidus*, and the fiddler crabs, *Uca pugnax* and *U. pugilator*, but not in the spider crab, *Libinia emarginata*. Mortality rates are negligible compared to those of animals induced to molt by eyestalk removal.

2. Precocious molts can be elicited in the land crab, *Gecarcinus lateralis,* by the loss of 5 to 8 appendages (pereiopods and chelipeds) but the loss of all 10 appendages inhibits molting. Loss of a cheliped which in *Gecarcinus* may have a mass ten times greater than a pereiopod is no more effective than loss of a walking leg.

3. The size of the regenerates formed in *Gecarcinus* is reduced by one-third from normal size when from 1 to 6 pereiopods are lost, and by one-half when 8 limbs are regenerated.

4. When one or more partially regenerated limbs is removed before a certain critical time in the premolt period (Stage D_0?) the animal re-regenerates replacement appendage(s). This results in significant lengthening of the interval before ecdysis occurs.

Note added in proof: Dr. George D. Bittner (personal communication) finds that in the crayfish, *Procambarus clarkii,* cutting of the nerves to 6 pereiopods is effective in shortening the interval between two ecdyses. In this species nerves to the pereiopods can be cut without the induction of autotomy.

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WATER-EXCHANGE IN THE CRAB *HEMIGRAPSUS NUDUS* MEASURED BY USE OF DEUTERIUM AND TRITIUM OXIDES AS TRACERS

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The water-permeability of several decapod crustaceans has been measured by Rudy (1967) using tritiated water (THO) as tracer. Among the animals studied was the Atlantic green crab, *Carcinus maenas*, a good osmotic regulator that has been the subject of many physiological investigations. Smith (1970) restudied *Carcinus maenas*, using deuterium oxide (DHO) as tracer, and made estimates of the water exchange rate and net water influx. Water-exchange rate was found to decrease with the salinity of the medium, as had previously been demonstrated for the very euryhaline crab *Rhithropanopeus harrisi* (Smith, 1967). Smith's results on *Carcinus*, based on DHO, seemed to indicate a markedly higher water-exchange rate than did the results of Rudy (1967), based on THO as tracer. Even after making reasonable corrections for differences in temperature and in the size of experimental animals, there remained a discrepancy in the hourly water-exchange fraction, with the values based on DHO about 20% higher than those based on THO. In this connection it was considered possibly significant that water-exchange values for the prawn *Palaemonetes varians* by Rudy (1967) using THO averaged only half the value obtained by Parry (1955) using DHO. It was further noted that, in unpublished studies, Smith had obtained a comparable 20% discrepancy between the water fluxes calculated from studies on *Rhithropanopeus harrisi*, using DHO and THO as tracers in separate experiments. Taken together, the above three sets of experiments suggest an isotope effect, but no quantitative value should be assigned because of the chain of assumptions involved in the estimates.

In considering the plausibility of an isotope effect between DHO and THO, it is improbable that it could be attributed to differences in diffusion rate among isotopic molecules of water as such. The molecular dimensions of D_2O and H_2O are almost identical (Kavanau, 1964), suggesting similar molecular diffusion rates. Deuterium and tritium oxides in dilute solution are almost entirely in the form of DHO and THO, with molecular weights of 19 and 20, not greatly different from the weight of 18 for H_2O. Wang, Robinson and Edelman (1953) found that the diffusion coefficients of DHO and THO in H_2O did not differ significantly, but that the diffusion coefficient of H_2O^{18} was about 14% higher than the values for DHO and THO. Chinard and Enns (1954) found no difference in the rates of passage of DHO and THO across capillary walls in the dog, and Enns and Chinard (1956) likewise found no significant difference in the passage of THO and H_2O^{18} in this experimental situation. Nevertheless, Johnson and Babb find it necessary to observe (1956, page 442), "It is noted that serious disagreement exists among various investigations of the self-diffusion coefficients of water" (involving the use of THO, DHO, and H_2O^{18} as tracers). Paganelli and Solomon (1957), noting the results of Wang *et al.*, (1953), increased their estimates of H_2O fluxes into the red cell, calculated on the basis of THO fluxes, by 14% to compensate for the reported difference in diffusion coefficients. Thus the matter of differences in the diffusion rates of THO, DHO, and H_2O cannot be regarded as settled.

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A second possibility for explaining an isotope effect between the uptakes of DHO and THO is that these isotopes might themselves interact with biological membranes in such a way as to lower water-permeability. Kavanau (1964) points out that the structural order and degree of hydrogen bonding are greater in liquid D_2O than in ordinary water, and it may be inferred that the bonding of T_2O is even stronger. But the experiments upon crustaceans cited in the present paper involved the use of D_2O at a concentration of 5%, while T_2O was used at a concentration of only 0.1%, hence it is unlikely that a measurably lower uptake of THO could be attributed to effects of the tritium isotope upon the membranes themselves. Although it may be possible that different species of crustaceans can distinguish between the chemically identical DHO and THO and show different DHO/THO permeability ratios, no evidence for such is known to the authors. It would have been most desirable to restudy *Carcinus*, but this Atlantic species was not available.

If an isotope effect of the magnitude reported is not likely to have resulted either from diffusion rate differences among isotopic water molecules, or as a consequence of alteration of the permeability of the membranes involved, attention should be directed to possible reactions involving the atoms or ions of the hydrogen isotopes themselves. Protium, deuterium, and tritium differ in mass as 1:2:3. The mobility of the ions H^+, D^+, and T^+ in water is regarded as not simply the diffusion of these ions as hydrated particles among water molecules, but as a process of proton transfer (*cf.* Pimentel and McCllelan, 1960, page 254). In this process the isotopic ions of D_2O may exchange rapidly between water molecules with the formation of D^+, H^+, OH^-, H_3O^+, and H_2DO^+ ions. The transfer of hydrogen and its isotopes from molecule to molecule may be expected to be mass-dependent and, to the extent that it is involved in the penetration of deuterium and tritium tracers into animals, might account for an observed slower uptake of THO as compared to DHO. In consequence, the DHO tracer would indicate a higher rate of water exchange than is indicated by THO, and the actual exchange of H_2O might be higher than is indicated by the use of either of the heavier isotopic tracers. Until such problems are clarified and a better quantitation made of any possible H_2O/DHO/THO isotope effect, water-permeability values obtained by use of different isotopes of water cannot be directly compared and the possibility must be borne in mind that neither DHO nor THO may indicate the full value of the permeability of animal surfaces to H_2O.

In order to clarify the discrepancy between our results (Rudy 1967, Smith

1970), we have carried out a double-tracer study with DHO and THO. This should eliminate variables of temperature and season as well as of size, sex, and moult stage of the animals used, and should minimize individual operational variation. We hoped that these experiments would allow us to quantify an isotope effect if such existed, or to rule it out as experimental artifact, and to provide an empirical check on the results given by the THO and DHO methods of determining water fluxes as we have used them.

MATERIALS AND METHODS

This joint study was carried out in late August and early September, 1970, at the Oregon Institute of Marine Biology, University of Oregon, Charleston, Oregon. *Hemigrapsus nudus* (Dana) was selected as the most readily obtainable euryhaline crab, not unlike *Carcinus* in its ecological preferences, and available in a wide size range. Experimental animals were maintained in running seawater (SW) at 12–13° C, or in 8-inch fingerbowls of SW diluted with tap water and kept cool in running SW. The local SW, taken near the mouth of the estuary of the Coos River, had an osmotic concentration of 1009 milliosmoles at the start of the study, dropping with the onset of rains to 960–980 milliosmoles. The osmotic pressures of blood serum and experimental media were determined by use of a Hewlett-Packard Vapor Pressure Osmometer, model 302B, with NaCl standard of 1000 milliosmoles. Blood was collected in amounts of 0.7 to 1 ml with a fine-tipped glass pipette, and discharged into a very small test tube, which was stoppered and placed in boiling water for 10 seconds. The firm white clot which formed was broken up with a slender glass rod, and the sample centrifuged to make the clear serum available. The blood of *T. nudus* clots so strongly that attempts to carry out VPO determinations on whole blood were unsuccessful. Determinations of osmotic pressure of serum as a function of external salinity were carried out on randomly selected crabs not used for the DHO-uptake studies, since the presence of even a small percentage of DHO, as a second volatile solvent, renders VPO determination impossible.

For studies of uptake of DHO and THO, 5% by volume of D_2O (Bio-Rad Laboratories, Richmond, California, 99 moles %) was added to the SW (giving "95% SW"), or to SW diluted with glass-distilled water (DW) so as to approximate an osmotic concentration of 600 milliosmoles ("60% SW"). To each liter of the above "5% D_2O" solutions was added 1 ml of tritiated water (THO) to given an activity of 1 μc per liter.

Exposure of crabs to the above THO/DHO solutions was carried out by one of us (R. I. S.) following as closely as possible the procedure used by Smith (1970) on *Carcinus maenas*. Tests were made in each salinity, at 10° and 20° C. Temperature variation was held within limits of ± 0.25°. Exposures were for 15 minutes, at the end of which each crab was rinsed quickly in isotope-free medium, wrapped and blotted in absorbent paper, and a blood sample drawn by puncture of the arthrodial membrane at a leg base. A few drops of this sample were immediately placed in the large end of a Pasteur pipette and distilled at 50° C, as in the experiments on *Carcinus* (Smith, 1970) and as described in detail in Welsh, Smith, and Kammer (1968), for determination of DHO. This, and subsequent calculations of the rate of DHO-uptake as a function of body weight, the determination of the hourly water-exchange fraction (K) on the basis of DHO-uptake, and the determination of Q_{10} for DHO-uptake were carried out by R. I. S. The remainder of the blood sample was discharged into a small screw-capped jar and frozen. These samples were then vacuum-distilled by P. P. R. in a freeze-drying apparatus, as nearly as possible following the method he had used in his study of *Carcinus* (Rudy, 1967), and the THO-saturation (% specific activity) of the blood determined with a Nuclear Chicago scintillation counter. Further treatment of these data was carried out by R. I. S. in order to eliminate any slight methodological differences.

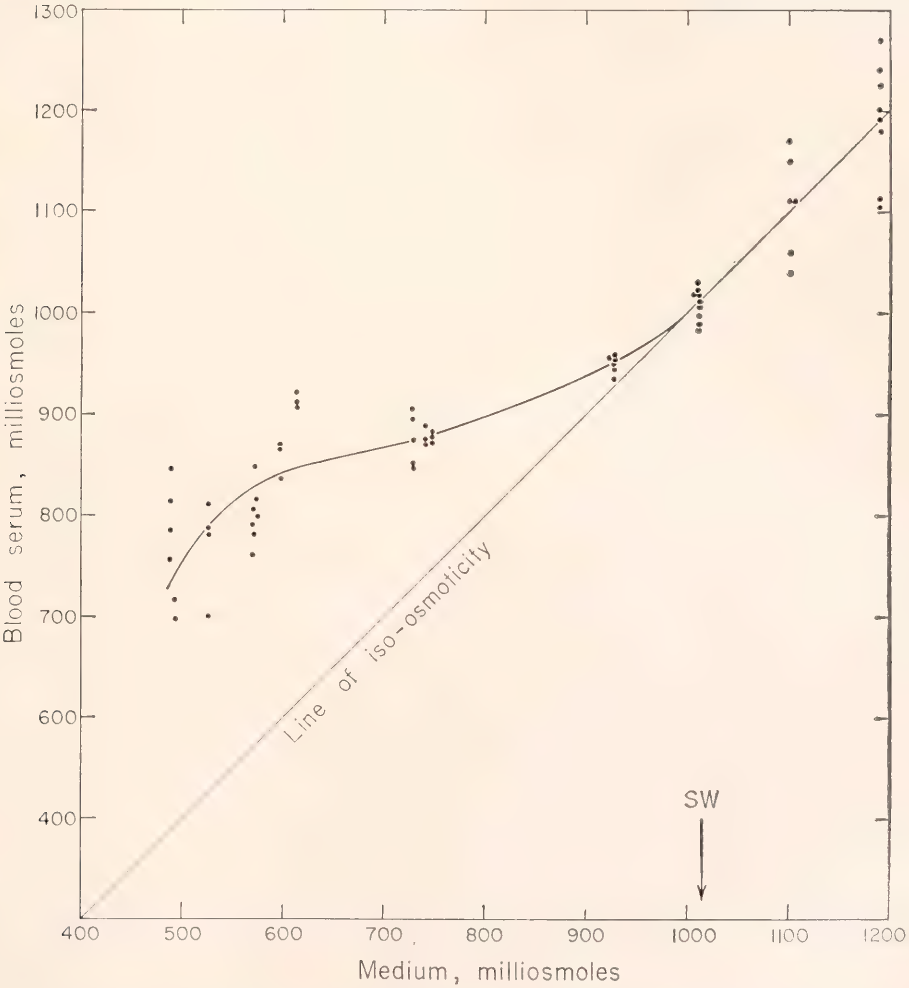

FIGURE 1. Osmotic concentration (milliosmoles) of blood serum of *Hemigrapsus nudus* as a function of concentration of the medium.

RESULTS

(*a.*) *The osmotic pressure of blood serum as a function of salinity*

One of the discrepancies between the results of Rudy (1967) and Smith (1970) regarding water-uptake in *Carcinus* lay in the fact that the authors found different concentrations of chloride in animals adapted to SW, and a relationship between chloride concentration and osmotic pressure of blood had to be assumed on the basis of possibly inappropriate data of earlier authors. VPO determinations of osmotic concentration of serum were agreed upon as a better basis for the estimation of the mole fractions of water in blood of animals adapted in 60% and 95% SW. The results (Fig. 1) show that the blood of *Hemigrapsus nudus* is iso-osmotic to the medium at about 980 milliosmoles (*ca.* 98% SW) under the conditions of the experiments, and hypertonic (*ca.* 865 milliosmoles) to the medium (*ca.* 600 milliosmoles) in 60% SW. From 100% to 120% SW, the blood is iso-osmotic with the medium. Animals in 50% SW showed signs of being over-

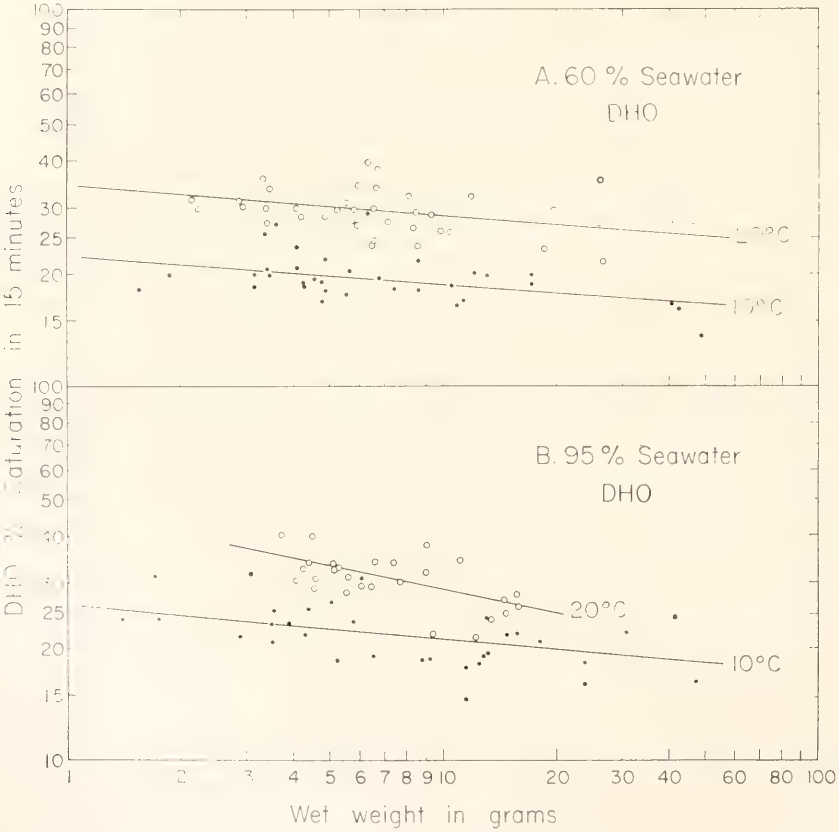

FIGURE 2. Uptake of DHO by *Hemigrapsus nudus* in a 15-minute exposure, as a function of salinity, temperature, and body weight, expressed as per cent of the concentration of DHO in medium.

stressed, some deaths occurred, and the curve of osmotic regulation showed a noticeable drop below the level of the regulatory plateau. 60% SW, as used in the isotope-uptake tests, appeared to be within the physiologically acceptable regulatory range.

(*b.*) *The uptake of DHO*

The concentration of DHO attained in the blood in a 15-minute exposure in 60% and 95% SW was determined at 10° and 20° C. Animals in three of the groups numbered 34, 41, and 31, with wet weights ranging from less than one to more than 40 grams. Plots of uptake against weight (Fig. 2 and Table I) yielded curves of comparable slopes (b-1 averaging − 0.0766). However, in one group, numbering 27 and tested in 95% SW at 20° C, the weight range was only 3.7 to 16 grams. The slope (b-1 = − 0.2091) is quite different from the rest, and is

TABLE I

Part A: Uptakes of THO and DHO by Hemigrapsus nudus *under four experimental conditions, with probabilities of differences in uptake related to type of isotope being significant ("t" test), calculated for wet weight of 10 g. Part B: Significance of differences in uptake in respect to salinity*

Part A

Experimental conditions		n	Isotope	Mean uptake as % Sat 15 min	Standard deviation	Probability
95% SW	20° C	27	THO	29.92	±3.69	Not significant
			DHO	29.66	±4.16	
	10° C	31	THO	20.93	±3.76	Not significant
			DHO	21.51	±3.46	
60% SW	20° C	41	THO	26.73	±4.34	>0.05
			DHO	28.50	±3.90	
	10° C	34	THO	17.24	±2.68	<0.02
			DHO	18.81	±2.56	

Part B

Salinity	Temp.	n	Isotope	Probability	n	Isotope	Probability
95% SW	20° C	27	DHO	>0.10	27	THO	<0.01
60% SW		41			41		
95% SW	10° C	31		<0.01	31		0.001
60% SW		34			34		

considered unreliable because of the small weight range tested. Therefore, the mean (b-1) value of the other three groups was used in adjusting the uptake values of the small fourth group to 10-g weight. This irregular procedure does not, in fact, change the mean value at 10-g weight very greatly, raising the mean uptake from 28.31% to 29.66% saturation.

When the uptake values of DHO (as % external DHO concentration or "% saturation") attained in 15 minutes are adjusted for a weight of 10 grams, which is not far from the mean weight of the crabs used, the values for DHO uptake are as shown in Figure 5 and Table I. A significant reduction of uptake is shown in 60% SW as compared to 95% SW at 10° C, confirming the apparent reduction of permeability to water (more propertly, reduction of water exchange) shown at lower salinities by the crabs *Carcinus* (Smith, 1970) and *Rhithropanopeus* (Smith, 1967). However, the reduction of water-exchange in 60% SW when measured at 20° C is not statistically significant. Probably this results from the inadequacy of the sample tested at 20° C in 95% SW. Mean hourly water exchange fractions "K," given by the equation $K = (2.3/t) \text{Log}_{10} (100/100\text{-}\% \text{ Sat})$, calculated from the mean DHO uptakes adjusted for a wet weight of 10 g are given in Table II, as are Q_{10} values for DHO uptake estimated by the ratios of "K" at 20° C to "K" at 10° C. Since the sample tested at 20° C in 95% SW is open to question, the Q_{10} value of 1.61 for DHO uptake in 60% SW is considered the more reliable of the two.

TABLE II

Hourly exchange fractions (K) and Q_{10} of DHO and THO uptake by Hemigrapsus nudus, *calculated for a wet weight of 10 g*

Experimental conditions		n	Isotope	Hourly exchange fraction "K"	Q_{10}
95% SW	20° C	27	DHO	1.41	[1.45]
	10° C	31		0.97	
60% SW	20° C	41		1.34	1.61
	10° C	31		0.83	
95% SW	20° C	27	THO	1.42	[1.51]
	10° C	31		0.94	
60% SW	20° C	41		1.24	1.63
	10° C	31		0.76	

(*c.*) *The uptake of THO*

The concentration of THO attained in the blood in 15-minute exposures in 60% and 95% SW was the result of the same exposures as for DHO. Plots of uptake against weights (Fig. 3 and Table I) yielded curves with (b-1) values much like those for DHO. One high value (– 0.1486) is regarded as unreliable because of the small weight range of the crabs used in this group (95% SW at 20° C). The average of the remaining three groups was – 0.0705, and this was used in correcting uptake in the deviant group to that of 10-g animals.

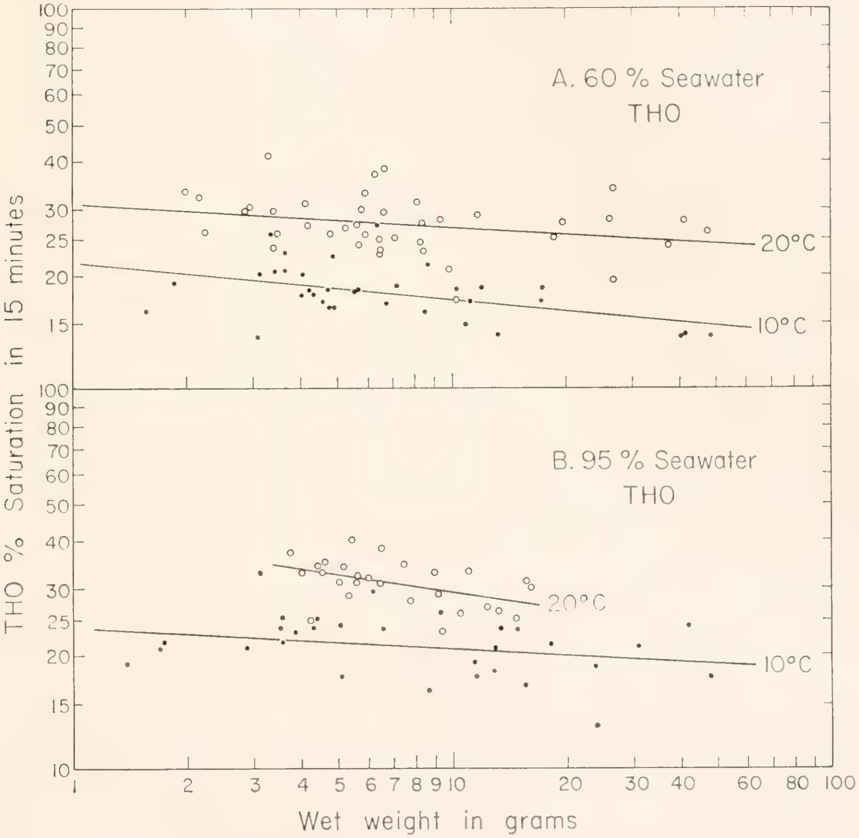

FIGURE 3. Uptake of THO by *H. nudus* in a 15-minute exposure, as a function of salinity, temperature, and body weight.

When the uptake ("% saturation") values attained in 15 minutes are adjusted for a weight of 10 g, the values for THO uptake are as shown in Figure 5 and Table I. A significant reduction of uptake is shown in 60% sea water as compared to 95% sea water in the tests, both at 10° and 20° C.

The mean hourly water exchange fractions (K), adjusted for a wet weight of 10 g are given in Table II, together with Q_{10} values, estimated as before. If we exclude the values from 95% SW because of the questionable validity of the group tested at 20° C, the value of 1.63 may be taken as the Q_{10} of THO uptake. This corresponds well with the value of 1.61 obtained for DHO in 60% SW.

(*d.*) *Comparison of DHO and THO uptake*

In three groups out of four, the THO method resulted in lower uptake values than were obtained by the DHO method (Fig. 4, 5). Both methods showed a significant reduction in water exchange at the lower salinity, with the exception of the DHO, 20° C, pair. Using the slopes of the other three groups as representing the actual relationship between salt concentration and water turnover, it would appear that the uptake determined by the DHO method in 95% SW at 20° is out of line and may be rejected.

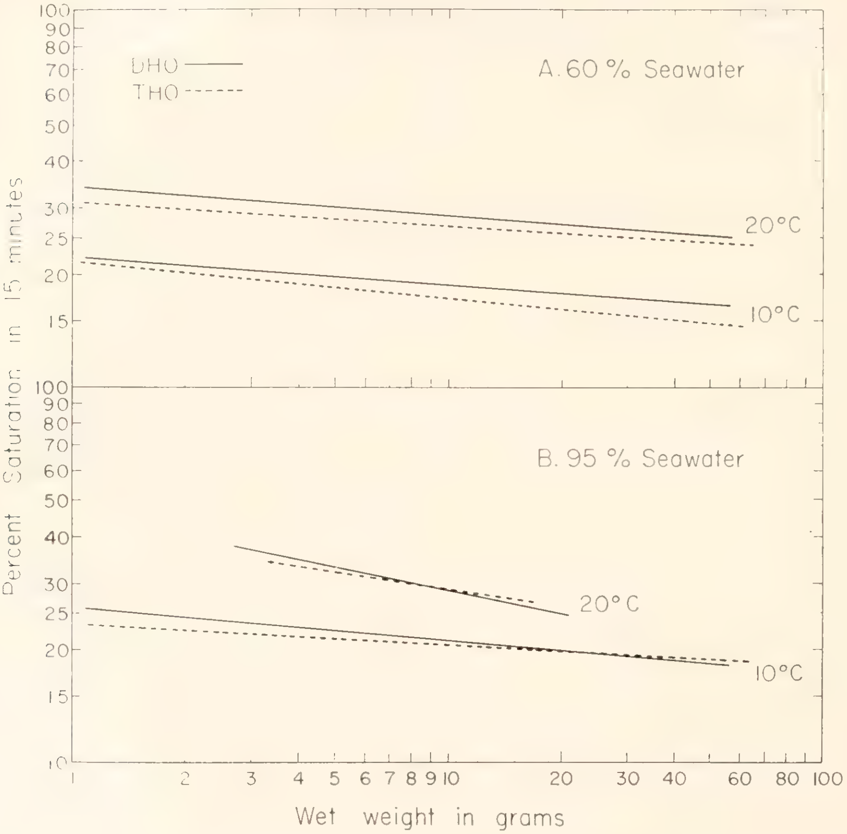

FIGURE 4. Comparison of uptakes of DHO and THO by *H. nudus* in respect to salinity, temperature, and wet weight, to show general smiliarity.

The ratios of K (THO)/K (DHO) derived from the other 3 groups average 0.94, that of the aberrant group being 1.01. The "t" indicates no significant difference between THO and DHO uptakes (as % saturation adjusted to 10 g wet weight) obtained in 95% SW at either 10° or 20° C (Table I). In 60% SW, significance is indicated by a probability greater than 0.05 at 20° C, and less than 0.02 at 10° C. The lack of significance within some groups may reflect low "n" values (41 or less).

However, when the ratio of THO/DHO uptakes is calculated for all paired determinations, involving all 4 groups, totaling 133 animals, the mean uptake of THO is 95.98% that of the DHO uptake. Even including the group tested in 95% SW at 20° C, which was "out of line," this value of 96% is significantly different from 100% at the 1% level of probability (99% confidence limits), and it is concluded that, in our experiments, the measured uptake of THO is significantly lower than that of DHO.

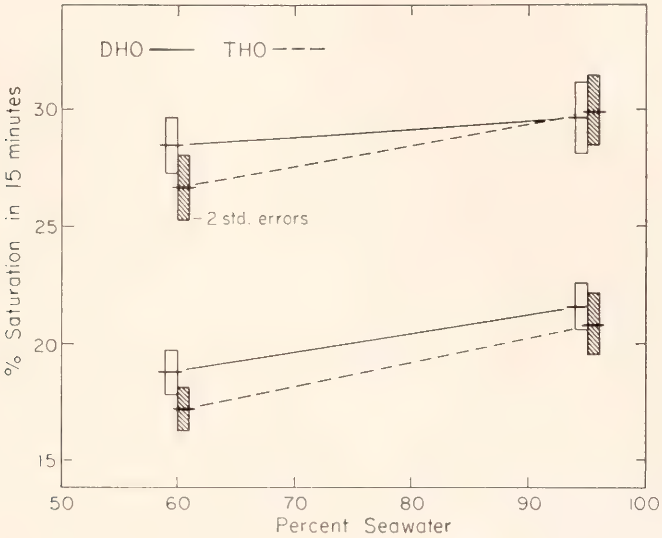

FIGURE 5. Uptake of DHO and THO by *H. nudus* as a function of salinity and temperature, adjusted for a wet weight of 10 grams. Blocks indicate 2 standard errors above and below the means.

DISCUSSION

But the actual meaning of this "significantly lower" uptake of THO in relation to DHO is not so easily determined. That the procedures used gave a small difference is reasonably clear. It is equally clear that the previously-reported difference of 20% (Smith, 1970) is not substantiated. Further refinement of procedures would probably reduce the scatter evident in the present results—in particular, increase of the exposure time so as to achieve saturations of ± 50% would be desirable. The 15-minute exposure was selected on the assumption that *Hemigrapsus nudus* would have a permeability comparable to that of *Carcinus,* but it proved to be less permeable than expected (*Carcinus:* K (DHO) = 2.07 in 50% SW at 18° C, Smith 1970; *H. nudus:* K (DHO) = 1.34 in 60% SW at 20°, this study).

It is possible that the difference observed between DHO and THO uptake lay in some aspect of the distillation procedures: possibly giving a spuriously high value for DHO expressing itself in the density determinations, possibly giving a spurious low value for THO as a result of undetected contamination (dilution) with atmospheric water in the vacuum distillation.

An attempt was made to make a second determination of DHO upon the vacuum-distilled samples, but this was unfortunately delayed for some weeks and, because many of these samples were contaminated in such a way as to raise their density, the results had to be discarded. The evidence for an isotope effect is not conclusive. We can conclude that the methods of the respective authors for DHO and THO determinations have yielded differing results suggestive of an isotope effect. But while it may be expected that adherence to a single method of distillation may make our procedures more alike, the fact that different final steps must be used to determine concentrations of the radio-active THO and the non-radioactive DHO makes conclusive demonstration of an isotope effect unattainable by our methods.

Despite this difficulty, we have in fact obtained different measures of water flux on the same experimental animal and, if we accept the possibility of an isotope effect, it follows that the actual flux of H_2O may be higher than that indicated by heavier isotopes. If a mass difference of 3:2 (T:D) results in fluxes in the proportions of 96:100, then a mass difference of 2:1 (D:H) might be expected to produce a difference in fluxes as great as 100:105.3. In other words, the relative uptakes of THO, DHO, and H_2O might be as *ca.* 91:95:100.

These differences in uptake as indicated by different isotopic tracers are not great, but may partly explain why measured values of urine production in crabs have in some cases been greater than those predicted from water-exchange measurements, as, for example, the urine volumes measured in *Carcinus* in 30% SW by Shaw (1961) and Binns (1969), as compared to the net influx values calculated on the basis of THO exchange (Rudy, 1967) and DHO exchange (Smith, 1970). In Table III (line 11) is shown a calculation of the daily net influxes of water at 20° C (presumed equal to urine volume) in *Hemigrapsus nudus,* calculated on the above assumption that uptakes of THO:DHO:H_2O are in the proportions of 91:95:100. These values are less than comparable values reported for *Carcinus* (line 12), indicating that *Hemigrapsus nudus* has a lower net water influx and should produce less urine than *Carcinus.* The values for net water influx in *Carcinus* in 60% SW as previously determined by Smith (DHO) and Rudy (THO) are caluculated and shown in line 12 (Table III), together with urine volume as estimated by H_2O-clearance methods by Shaw (1961). These values show an overall inverse relation to isotope weight consistent with the present hypothesis of an isotope effect. This trend is less clear in the case of 95% SW, but it may be noted that the net influx value from Rudy (THO) and urine volume from Shaw (clearance) had to be obtained by interpolation. The results are only consistent with the concept of an isotope effect; they do not prove it. What they show is that the THO method for some reason tends to indicate a low value for water flux relative to the DHO method, and that measures of urine volume in crabs by methods not involving the heavier isotopes of water may yield higher values. However, the difference between urine volumes as determind by methods not involving water isotopes, and the net influxes calculated from water-isotope exchange studies, is still too great to be explained solely on the basis of an isotope effect as small as the 5–10% suggested here. Such factors as unstirred layers or pores may also be involved. And, obviously, studies by several methods used upon the same species should furnish more satisfactory data than is currently available.

TABLE III

(Lines 1-11) Calculation of water influxes in Hemigrapsus nudus *at 10° C in 60% and 95% SW according to hourly water exchange (K) values based upon simultaneous THO and DHO uptake, with estimates of net H_2O influx. Water content assumed to be 70%.*
(Line 12) Published comparable values for Carcinus

1. Medium, % SW and conc. milliosmoles	60 (600 millismoles)			95 (950 millismoles)		
2. Concentration of blood, milliosmoles	845			965		
3. Conc. of blood as % SW	84.5			96.5		
4. Mole fraction water of medium	0.9893			0.9832		
5. Mole fraction water of blood	0.9850			0.9829		
6. Mole fraction difference	0.0043			0.0003		
7. Hourly water exchange fraction (K) at 10° C	THO	DHO	H_2O (est.)	THO	DHO	H_2O (est.)
	0.76	0.83	*ca.* 0.855	0.94	0.97	*ca.* 1.025
8. Daily water influx (K × 70 × 24) as % body weight per day	1276.8	1394.4	1436.4	1579.2	1629.6	1722.0
9. Daily *net* water influx as % of total influx	0.435	0.435	0.435	0.0305	0.0305	0.0305
10. Daily *net* influx as % of body weight at 10° C	5.55	6.06	6.25	0.48	0.50	0.525
11. Daily net influx in *H. nudus* at 20° C (Q_{10} = 1.62)	8.99	9.82	10.1	0.78	0.81	0.85
12. Calculated daily net influx in *Carcinus* (DHO and THO) and urine volume (direct meas.)	net influx 9.4 Rudy (1967) recalc. 20°	net influx 14.5 Smith (1970) recalc. 18°	urine vol. 16.5 Shaw (1961) 16°	net influx 1.25 Rudy (1967) recalc. 20°	net influx 1.19 Smith (1970) recalc. 18°	urine vol. 5.0 Shaw (1961) 16°

SUMMARY

1. The crab *Hemigrapsus nudus* regulates the osmotic pressure of its blood in media down to less than 60% seawater, and is iso-osmotic in 100% seawater and higher salinities.

2. Measurements of simultaneous uptake of tritiated water (THO) and deuterated water (DHO) give uptake values for THO about 96% those obtained with DHO.

3. The Q_{10} of uptake of both isotopes is about 1.62, and the relation of uptake of both to body weight is similar.

4. The results are consistent with, but do not prove, the concept of a small isotope effect in the uptake of THO and DHO. Published reports of higher water fluxes based on methods not involving isotopes of water are consistent with the argument for an isotope effect. It is suggested that water fluxes based on methods using THO:DHO:H_2O as ordinarily employed are of the relative magnitudes 91:95:100.

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A COMPARISON OF *IN SITU* AND *IN VITRO* RESPONSES OF CRUSTACEAN HEARTS TO HYPOXIA[1]

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Although crustacean cardiac physiology has been extensively researched, there remain several aspects which have scarcely been investigated. One such area has been the response of the crustacean heart to deficiencies in oxygen.

An early indication of the cardiac response to hypoxia was noted by Burger and Smythe (1953) in the lobster *Homarus americanus.* It was observed that the heart of this animal slowed its rate of beating when the lobster was out of the water. In this situation the gill filaments would be collapsed and the effective exchange area reduced, thereby placing the animal in an hypoxic situation. Larimer (1962, 1964a, 1964b), in a series of studies on the crayfish *Procambarus simulans,* found that these animals demonstrate a marked bradycardia when the oxygen is driven from the water containing them. Thompson and Pritchard (1969) subjected the burrowing shrimp *Callianassa californiensis* to hypoxia and noted a significant reduction of heart rate at very low oxygen concentrations.

In the present investigation the response to hypoxia of the heart of the crab *Cancer magister* (Dana) will be described. Previous investigations of these responses have been limited to the response of the *in situ* heart. This report will also deal with the response of the isolated heart to hypoxia. The effect of lowered oxygen concentrations on amplitude or magnitude of contraction is also examined.

METHODS AND MATERIALS

This study was carried out at the Oregon State University Marine Science Center at Newport, Oregon and at the main campus in Corvallis.

Adult specimens of *Cancer magister* ranging in carapace width from 10 to 18 cm were collected from Yaquina Bay and maintained at 12° C.

All experiments were performed at 12 ± 1° C which approximates the temperature in the area from which the animals were collected.

Recording of heart rate and amplitude of contraction was achieved by the use of a Narco Bio-Systems physiograph in conjunction with a Narco Type B photoelectric force transducer-type myograph.

Oxygen concentration in the experimental containers was manipulated by bubbling nitrogen or air directly into the water or perfusion solution (in the case of the isolated hearts). A Yellow Springs Instrument Company Model 54 oxygen meter and Model 5420 oxygen probe were used to moniter the oxygen concentration in the testing bath.

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[1] Supported in part by Institutional Sea Grant GH-45.

[2] This work was submitted by Daniel F. Stiffler to the Graduate School at Oregon State University in partial fullfillment of the requirements for the Master of Science degree.

Animals were prepared for *in situ* recording by inducing autotomy of their legs and then tied to a restraining board. Limb autotomy seemed to have no effect on the pattern of the recorded heart beat, as demonstrated by recordings of the heart beat of a few animals with intact legs. After the removal of a small piece of carapace overlying the heart, a bent pin was inserted into the still intact hypodermis and connected by a thread to the myograph. The oxygen probe and air stones were then positioned in the bath and the container was covered to restrict the entry of air.

The preparation for the *in vitro* heart beat recordings involved a modification of a method described by Welsh and Smith (1960). The pericardial cavity was carefully exposed and ligatures were tied around the posterior lateral ligaments of the heart. These later served as a convenient means of handling the heart. Next, the abdominal artery was cannulated with a piece of suitable sized polyethylene tubing. Finally the anterior arteries were ligatured and the heart was removed to the recording chamber and attached to the myograph. The recording chamber consisted of a 4 × 4 × 6 inch plastic refrigerator container equipped with air stones and an oxygen probe. The bottom of the chamber was pierced by a tube through which the perfusion solution passed and to which the cannula was attached.

After mounting the heart in the chamber, the rate of perfusion could be controlled by means of a Teflon needle valve. Shortly after initiating perfusion the heart resumed its rhythmic beat with a steady rate and amplitude. Before a preparation was judged usable it had to meet the following criteria: display of reasonably steady rate and amplitude; recovery to near normal rate and amplitude after hypoxic stress; and response to changes in perfusion rate with corresponding changes in rate and amplitude. An increase in perfusion rate has been shown to result in an increase in rate and amplitude for the heart of *Maia squinado* (Izquierdo, 1931).

All hearts were perfused with a solution identical to that used by Davenport (1941).

The same protocol was used for both *in situ* and isolated hearts in determining the response to hypoxia. After the recording instruments were attached the animal was allowed to stabilize at least ten minutes and the oxygen concentration of the water was recorded. Nitrogen was then passed through the water for a short period. After the heart beat and oxygen concentration had again stabilized the procedure was repeated. This was done until the oxygen concentration had decreased to below 1 mg O_2 per liter of sea water. Air was then re-introduced into the bath and the heart beat was allowed to return to normal. During the recovery period the oxygen concentration was periodically recorded in order to evaluate the latency of the response to oxygen.

RESULTS

The heart rates of the crabs were found to vary somewhat from animal to animal but were fairly consistent from one experiment to the next for a given animal. The mean rate for *in situ* hearts when beating at maximum was 79 beats per minute with a range of 72 to 92. The mean rate for isolated hearts at maximum was 54 beats per minute with a range of 37 to 81.

Since this investigation is primarily concerned with relative values the data are expressed as a per cent of maximum rate. To prevent the possibility of random accelerations of short duration from biasing the data, the maximum rate had to be sustained for a period of at least three minutes to be considered as valid. The resultant values are best termed relative rate.

The amplitude recorded by the myograph provides a rough index of the strength of contraction of the hearts. This is quantifiable in terms of relative amplitude. In some of the recordings amplitude varied slightly from contraction to contraction but was maintained at an overall consistent level. This was especially so in the case of the *in situ* hearts. To determine the relative amplitude under these conditions, the heights of ten consecutive peaks, chosen at random, were averaged. The myographic recording of the amplitude of contraction in *in situ* hearts is complicated by the complex attachments of the heart, both within the pericardial cavity and externally with the myograph. The attachment of the heart to the myograph in the *in situ* state is, of necessity, indirect due to the need to preserve integrity of the open circulatory system. To accomplish this the pin connected to the myograph was inserted in the hypodermis which, in turn, was indirectly connected to the heart. This left the open circulatory system intact but resulted in a complex transmission of the heart's action to the myograph. This did not affect the recording of rate but may have significantly altered the pattern of recorded amplitude. With the isolated hearts the myograph was connected directly to the hearts themselves. Since the amplitude response of the isolated hearts was

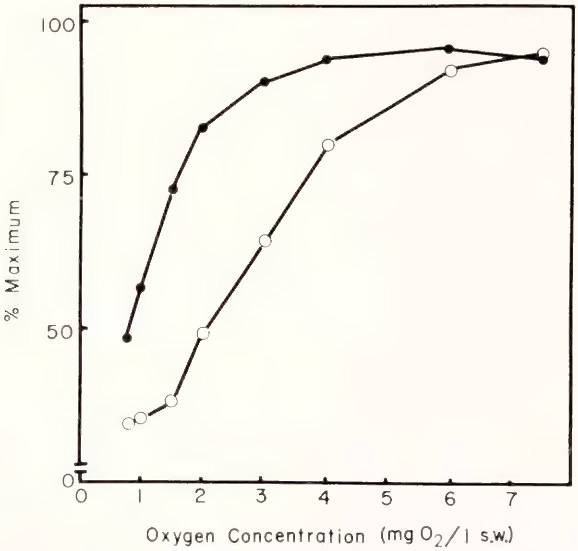

FIGURE 1. Relationship between heart rate (●) and amplitude (○) and oxygen concentration for *in situ* hearts. Each curve represents the results of eight experiments in which rate and amplitude were recorded simultaneously. The rates and amplitudes are expressed in terms of per cent of maximum while the oxygen concentration is in mg O_2/l sea water. The standard errors ranged from 0.8% to 6.0% for rate and from 2.0% to 8.1% for amplitude.

similar in nature to that of the *in situ* hearts the greater variability of the latter may have been due to the indirect means of recording.

A persistent feature of many *in situ* recordings was the occasional appearance of a transient cardiac arrest. This was observed to occur both in diastole (Fig. 3a) and in systole and was often seen either accompanying or just preceding limb or limb stub movement. Similar cardiac inhibition has been reported in *Asellus aquaticus,* an isopod (Needham, 1954), *Panulirus argus,* a lobster (Maynard, 1960) and *Procambarus clarkii,* a crayfish (Larimer and Tindel, 1966).

The responses of both *in situ* and isolated hearts to hypoxia were marked bradycardia and depression of amplitude. Although the responses were similar in most respects, certain differences were apparent. Figure 1 depicts the response of the *in situ* hearts to hypoxia and it may be seen that as oxygen concentration fell below 1 mg O_2/l both rate and amplitude decreased to less than 50 per cent of the values in air-saturated water. The pattern of this decrease, however, is different for rate and amplitude. While the decrease in rate is clearly hyperbolic, with the most significant decrease occurring between 1 and 2 mg O_2/l, the decrease in amplitude tends more toward linearity in this range of oxygen concentrations. The patterns of decrease in rate and amplitude of isolated hearts, however, are very much alike, both tending toward a hyperbolic relationship (Fig. 2). It is felt that the indirect means of recording *in situ* heart movements is responsible for the apparently anomalous pattern of *in situ* amplitude.

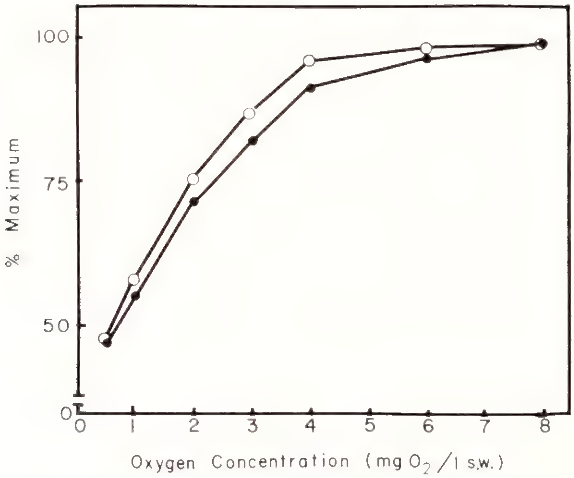

FIGURE 2. Relationship between heart rate (●) and amplitude (O) and oxygen concentration for isolated hearts. Each curve represents the results of six experiments in which rate and amplitude were recorded simultaneously. The rates and amplitudes are expressed in terms of per cent of maximum while the oxygen concentration is in mg O_2/l sea water. The standard errors ranged from 0.6% to 8.0% for rate and from 0.6% to 5.4% for amplitude.

The time course of the recovery of the hearts to near the maximum rate upon readmission of oxygen to the water after hypoxic stress differed greatly in *in situ* and isolated hearts. Nineteen experiments were performed and in each case the response of the *in situ* hearts to oxygen was much more rapid than was the response of the isolated hearts. The mean recovery time for *in situ* hearts was 22 seconds while the mean recovery time for isolated hearts was 127 seconds. These are significantly different at the 0.001 level (t-test). The mean rates of increase of oxygen concentration were roughly similar in the two situations being 1.82 mg O_2/l/min for *in situ* and 1.24 mg O_2/l/min for isolated preparations. Typical recordings showing these responses are given in Figure 3.

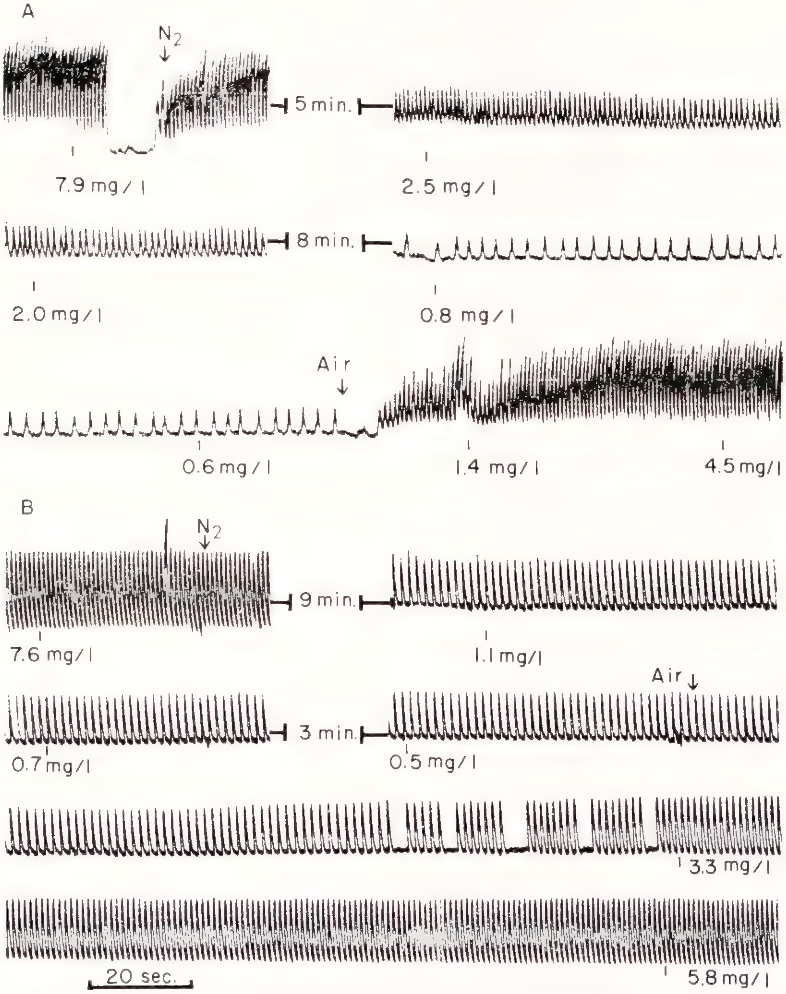

FIGURE 3. Recordings of typical *in situ* (A) and isolated (B) heart beats. The records are continuous except where breaks are indicated. Oxygen concentration at various points is given in mg O_2/l sea water.

As a precaution against the possibility that the response of the *in situ* hearts to aeration of hypoxic water may have been a mechanoreceptive one, associated with the turbulence caused by aeration, several crabs were subjected to sudden vigorous nitrogen bubbling while under hypoxic stress (Fig. 4). In no case was there a duplication of the aeration response.

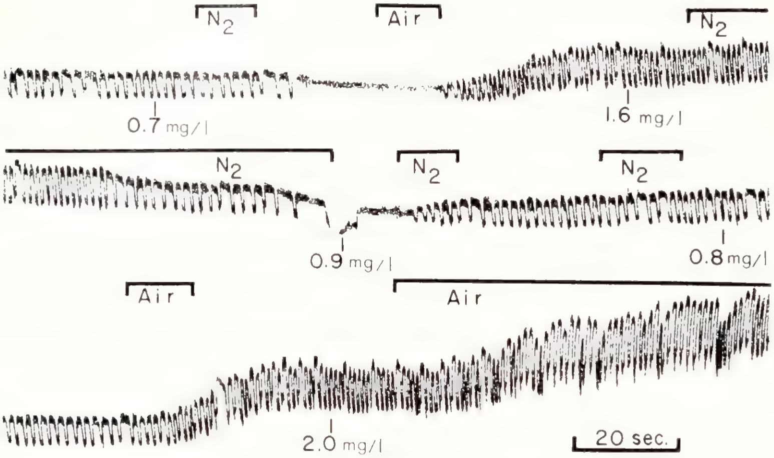

FIGURE 4. Continuous recording showing the failure of the *in situ* heart to respond to turbulence caused by vigorous nitrogen bubbling. The duration of aeration and nigration bubbling is shown above recording while oxygen concentration of the water is indicated below.

DISCUSSION

The variation in the maximum heart rate probably results from a number of factors. Since the animals were held for varying lengths of time without feeding, their nutritional states might have varied. More importantly, different levels of "trauma" following preparation of the animals undoubtedly had some effect on the level of heart rate and amplitude. It is felt, however, that since these parameters were quite stable within a given preparation, and since relative rates and amplitudes were used, the variability does not significantly alter the interpretations of the data.

A comparison of the mean maximum heart rates of the isolated and *in situ* hearts shows that the latter beat much more rapidly (79/min *vs.* 54/min). The difference may be due to the absence of the cardio-acceleratory nerves and possibly the pericardial organs in the isolated preparations. The pericardial organs are neurosecretory elements which have a pronounced cardio-excitatory effect (Cooke, 1964).

The bradycardia resulting from hypoxia confirms that observed in other crustaceans investigated. These include *Procambarus simulans* (Larimer, 1962, 1964a, 1964b) and *Callainassa californiensis* (Thompson and Pritchard, 1969).

The significance of the hypoxia-induced bradycardia is not clear. Larimer (1962) felt that it might be due to a high sensitivity of the heart muscle to lowered oxygen concentration. Subsequent work (Larimer, 1964a), however, indicates that this may not be the case, at least for *P. simulans*, as the per cent extraction of oxygen from the respiratory stream increased during hypoxia. To explain this

Larimer has hypothesized an increased rate of circulation, facilitated by an increased stroke volume and decreased peripheral resistance during bradycardia. A similar increased per cent extraction of oxygen has been reported for the lobster *Homarus vulgaris* during hypoxia (Thomas, 1954) and circulatory responses were also postulated as an explanation.

The increased circulation rate at low concentrations hypothesized by Larimer (1964a) might be advantageous for an animal such as *P. simulans,* whose respiratory pigment has a high affinity for oxygen. Larimer and Gold (1961) report a P_{50} of 3.5 mm Hg for this crayfish. *Cancer magister,* however, has a low oxygen affinity hemocyanin with a P_{50} of 19.6 mm Hg at 10° C (Johansen, Lenfant and Mecklenburg, 1970). *Callianassa californiensis* also has a high oxygen affinity hemocyanin with a P_{50} of 3–4 mm Hg at 10° (Miller and Pritchard, unpublished data), and shows a bradycardia at low oxygen concentrations (Thompson and Prithcard, 1969). Data regarding changes in per cent extraction of oxygen for *Cancer magister* during hypoxia are not available. However, Johansen *et al.* (1970) report an increase in the gradient between exhaled water and arterial blood oxygen during partial hypoxia which might reflect a decreased per cent extraction of oxygen from the respiratory stream. This, however might also be explained by a possible increased ventilation in these animals during hypoxia (Johansen *et al.,* 1970; Stiffler, 1970).

Cancer magister is capable of regulating its metabolic rate over a wide range of oxygen concentrations (Johansen *et al.,* 1970). It is noteworthy that the lower limits of this regulation correspond roughly to the range of oxygen concentrations in which the decrease in heart rate and amplitude, reported here, are most apparent. Regulation of metabolic rate over a wide range has also been observed in the shrimp *C. californiensis* (Thompson and Pritchard, 1969). Bradycardia in this species also occurred at oxygen concentrations roughly corresponding to the critical oxygen concentration at which metabolic rate began to decrease. These authors suggested that the maintenance of a constant heart rate aids this animal in regulating its oxygen consumption rate.

The decrease in amplitude of contraction observed in this study is difficult to interpret. The fact that it occurred consistently in isolated hearts as well as *in situ* hearts would seem in indicate that it is not an artifact of pin placement in the latter case. Although the amplitude of contraction is certainly not a reliable index of cardiac output, changes in this parameter may roughly indicate changes in stroke volume. If this is the case the increased stroke volume hypothesized by Larimer (1964a) for the crayfish does not appear to be operating in the case of *Cancer magister.* What may be happening is that as the oxygen concentration decreases below the range where the animals regulate metabolic rate and oxygen consumption declines, the circulation rate also decreases. The fact that the isolated hearts showed a bradycardia under hypoxic conditions indicates that the heart muscle may be sensitive to oxygen deprivation in this animal.

All of this suggests that an increased rate of circulation during hypoxic stress as proposed by Larimer (1964a) for crayfish is not a generalized response for decapod crustaceans. The present data, as well as that of Johansen *et al.* (1970) and Thompson and Pritchard (1969), suggest that the bradycardia may be accompanied by a decrease in circulatory rate in at least some decapods. In any case the interactions involved in gas exchange cannot be fully assessed for *Cancer magister* until measurements of cardiac output and per cent extraction of oxygen from the respiratory stream have been made under conditions of varying oxygen concentration.

The response of the isolated hearts to hypoxia was quite similar to that of the *in situ* hearts. The correlation between changes in amplitude and rate was much closer in the case of the isolated hearts, however. For the *in situ* hearts the relationship between heart rate and oxygen concentration was clearly hyperbolic, whereas the relationship between amplitude and oxygen concentration tended more toward linearity (Fig. 1). This might be explained by the indirect connection between the heart and the myograph discussed above. With the isolated hearts, both functions tended toward a hyperbolic relationship (Fig. 2). This might be expected with the more direct means of recording the actions of the isolated hearts.

One of the more interesting differences between the isolated and *in situ* hearts' responses lies in their respective patterns of recovery. While the *in situ* hearts recovered quite rapidly, within a few seconds of aeration of hypoxic water, the isolated hearts were quite slow to recover, consistently taking several minutes to do so (Fig. 3a and 3b). As there is no way of instantaneously restoring the oxygen to the water the dead time involved in aeration may be significant.

A possible explanation of the discrepancy in these latencies is that the *in situ* heart remained under the control of the animal's nervous system. This would be suggestive of a receptor or receptors sensitive to oxygen. Such hypothetical receptors have been implicated in the responses of other arthropods to oxygen (Waterman and Travis, 1953; Larimer, 1964a; Farley and Case, 1969; Gamble, 1971). The possibility that the response might be due to the turbulence associated with aeration can be ruled out as vigorous bubbling of nitrogen, initiated suddenly in still, hypoxic water, did not elicit the response (Fig. 4).

Although the idea of oxygen receptors is an intriguing one, until such receptors are found and action potentials recorded from their nerves at varying oxygen concentrations, their existence must remain highly speculative.

SUMMARY

1. Heart rate and amplitude were recorded for both *in situ* and isolated hearts of *Cancer magister* exposed to lowered oxygen concentrations.

2. Both rate and amplitude declined markedly as the oxygen was driven from the water. This occurred in both *in situ* and isolated hearts.

3. The recovery of *in situ* hearts to near normal rate and amplitude occurred quite rapidly upon the readmission of oxygen to the water.

4. Isolated hearts recovered very slowly to aeration of hypoxic water.

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ACTION OF HYDROSTATIC PRESSURE ON SEA URCHIN CILIA[1]

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Hydrostatic pressure has been shown to affect organized cellular structures and macromolecular synthesis (Zimmerman, 1970). In several different species of protozoa (Kitching, 1957, 1970) and in marine tissue (Flugel and Schlieper, 1970), it has been reported that ciliary activity is inhibited by pressure. Pressure causes the disappearance of microtubular arrays in structures such as the mitotic apparatus (Zimmerman and Marsland, 1964; Zimmerman and Philpott, unpublished) and the axopodia of *Actinosphaerium* (Tilney, Hiramoto and Marsland, 1966). In *Tetrahymena* the proximal portions of the central ciliary fibers and longitudinal microtubules are affected by pressures of 7500–10,000 psi (Kennedy and Zimmerman, 1970). However, fine structural analysis of the cilia and sperm flagellae of sea urchin embryos indicate that these structures are resistant to pressure treatment (Tilney and Gibbins, 1968, 1969; Marsland, 1970).

In protozoa, protein synthesis is essential for complete flagellar and cilia regeneration (Rosenbaum and Child, 1967; Rosenbaum, Moulder and Ringo, 1969); however, RNA and protein synthesis are not necessary for initial cilia formation and regeneration in sea urchin embryos (Auclair and Meismer, 1965; Auclair and Siegel, 1966). The sea urchin thus provides a relatively simple system for studying processes involved in the organization and assembly of the cilium.

The experiments reported here were designed to investigate the effects of hydrostatic pressure on both the intact cilium and the regenerating cilium in the sea urchin embryo.

METHODS AND MATERIALS

Embryos of *Arbacia punctulata* were maintained in natural seawater; embryos of *Strongylocentrotus purpuratus* were kept in artificial seawater (Instant Ocean). Eggs and sperm were shed by KCl injection or electrical stimulation (Harvey, 1956). After insemination development was allowed to proceed at 18° C for both species.

Deciliation was performed according to Auclair and Siegel (1966). Hypertonic seawater treatment (29.2 g NaCl/liter of seawater for 1–2 min) was found to remove essentially all cilia. Following deciliation embryos were resuspended in normal seawater for regeneration to occur.

The initial stages of regeneration were observed and measured after staining with Lugol's iodine. Measurements on cilia longer than 6–8 μ were made on living specimens (in 5% methyl cellulose) using phase contrast optics. The longest observable cilium on each of five embryos was measured and the values averaged.

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[1] Work supported in part by National Research Council of Canada.

[2] Predoctoral Fellow National Research Council of Canada.

The temperature-control housing, pressure pump and microscope-pressure chamber were similar to those described by Marsland (1950). Following deciliation the embryos at the stage of swimming blastula or early prism were transferred to a Lucite chamber which was then placed inside the main pressure vessel. In all regeneration experiments, the pressure (2000–10,000 psi, which is equivalent to 139–680 atm, or 137.8×10^5–689×10^5 Newtons/m^2 or Pascals) was applied 3 min after deciliation. Non-pressurized control embryos were placed in similar chambers but remained at atmospheric pressure. The embryos were observed at magnifications up to 600 × while the cells were under pressure.

RESULTS

Preliminary observations

The pattern of behavioral changes induced by pressure on swimming embryos of *Strongylocentrotus purpuratus* and *Arbacia punctulata* is dependent upon both magnitude and duration of treatment. The changes induced at the higher pressures are quite clear whereas at lower magnitudes of pressure there is considerable individual variation within a single group.

Strongylocentrotus embryos (swimming blastulae) were subjected to varying magnitudes of pressure (6000–10,000 psi) while under continuous observation. Within two minutes after compression to 10,000 psi, the embryos began to slow down, although they still maintained their normal spiralling motion. Following 3–5 min of compression the embryos began to collect on the bottom of the pressure chamber where they rotated with one pole against the surface. The rate of spinning gradually slowed and was replaced by a very rapid vibratory motion. After 7–8 min most of the embryos were vibrating, however a few embryos retained their spinning motion and a few were completely motionless. After 10–12 min essentially all of the embryos were motionless.

When the pressure was lowered to 8000 psi the cells responded in a similar fashion to that found at 10,000 psi except that some motion was evident 15 min after compression. At 7000 psi it took considerably longer for the embryos to display the previously described effects. After 10 min at 7000 psi most of the embryos were still swimming, albeit more slowly than normal, and only a few embryos had settled to the bottom of the chamber. By 20 min all of the embryos had settled and were rotating slowly. Thirty min after compression, the embryos were still rotating although they were beginning to show signs of disaggregation. For this reason, longer durations were not employed at this pressure level. With a further reduction in pressure to 6000 psi it required between 20 and 30 min for most of the embryos to settle on the bottom of the chamber. At 50–60 min after compression, most of the embryos were still rotating. It was felt that the extent of disaggregation limited the usefulness of experiments of longer duration than 60 min.

In experiments in which the pressure was released while the embryos were still active (*i.e.,* displaying some movement) an initial burst of activity followed immediately upon decompression.

It was generally found that *Arbacia* embryos displayed similar behavior; however, they were more sensitive to pressure treatment than *Strongylocentrotus* embryos.

Pressure effects on sea urchin cilia

From the above study it was evident that high pressure treatment resulted in a loss of cilia. In order to investigate this phenomenon, *Strongylocentrotus* embryos were subjected to pressure and following decompression they were studied by phase contrast microscopy. The number of cilia lost depended partly on the magnitude and partly on the duration of treatment. At pressures of 8000 psi and above more than 90% of the cilia were removed within 10 min; at 7000 psi more than 80% of the cilia were removed 30 min after compression. When the pressure was reduced to 6000 psi more than 50% of the cilia were lost in 45–60 min. The cilia were lost as apparently intact structural units and could be observed floating in the medium. The detached cilia had distended bulbous distal tips similar to the cilia which remained on the embryos.

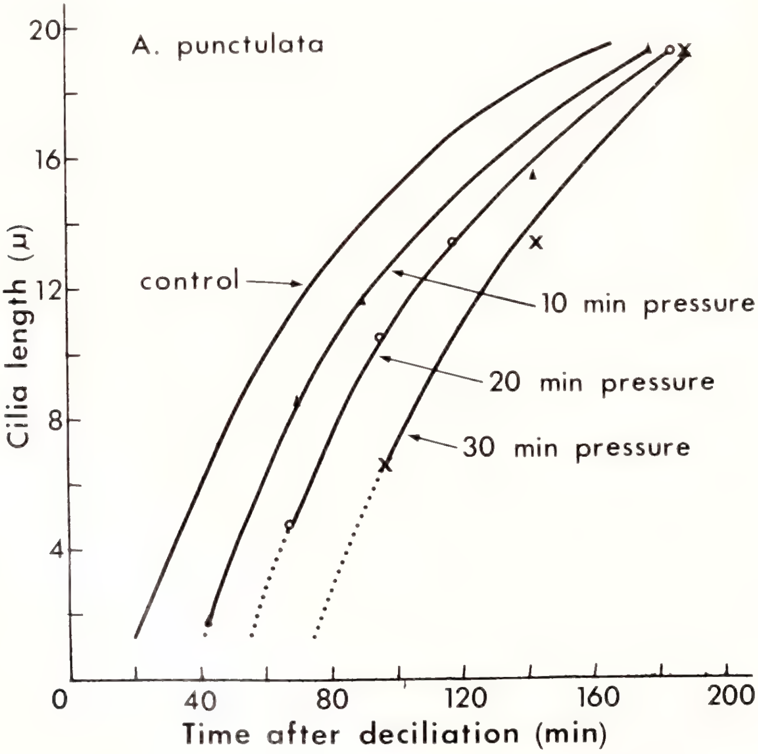

FIGURE 1. Duration of pressure treatment and cilia regeneration in *Arbacia punctulata*. Sea urchin prismatic stage larvae were deciliated and subjected to 10,000 psi for 10, 20 and 30 min of compression. At various times after decompression the lengths of the cilia were measured.

In a series of experiments at 10,000 psi, in which groups of embryos were treated for varying durations (2–10 min), it was found that a few cilia remained on those embryos still undergoing the vibratory motion and essentially all cilia were gone from those embryos in which all motion had ceased.

In embryos treated for long durations (30–60 min) at lower pressures (6000 psi) the apical tuft cilia were found to be more resistant to pressure than other cilia. In embryos displaying partial cilia loss, the deciliation frequently occurred in a patch near the vegetal pole.

Comparable studies with *Arbacia* embryos indicated that they were more sensitive to pressure-induced cilia loss than *Strongylocentrotus* embryos. In some experiments, pressures as low as 2000 psi for durations of less than one hour caused essentially total loss of cilia from *Arbacia* embryos.

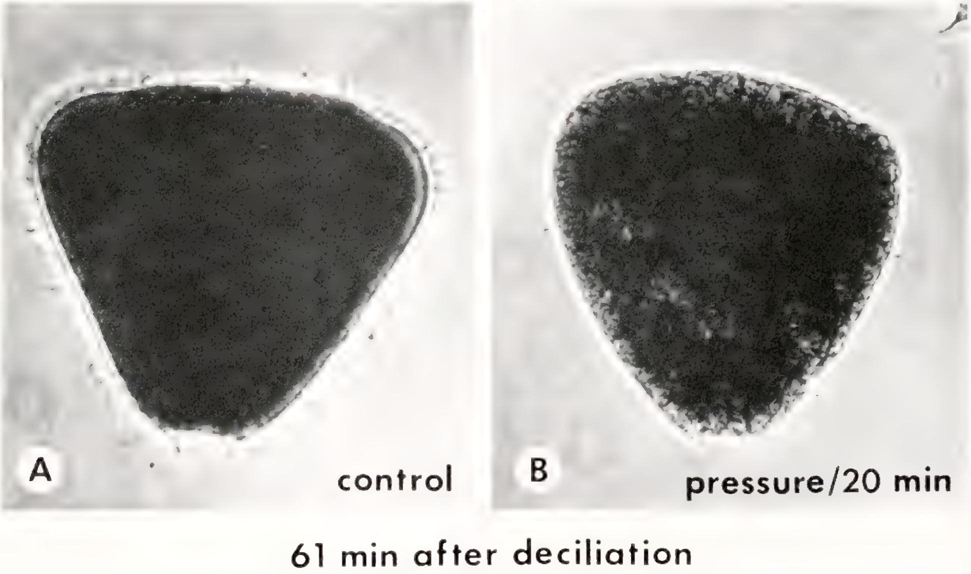

FIGURE 2. Pressure induced delay of cilia regeneration. The photomicrographs were taken 61 min after deciliation by hypertonic seawater; (A) Control embryo; (B) Experimental embryo was subjected to 10,000 psi for 20 min. Note the rounded appearance of the surface cells. Both control and experimental embryos were stained with Lugol's iodine.

Pressure effects on cilia regeneration

Since it is well established that high pressure interferes with microtubule polymerization, the effects of pressure on cilia regeneration were investigated. The two parameters studied were the magnitude and the duration of pressure treatment. Embryos were deciliated by hypertonic sea water treatment and subjected to compression (2000–10,000 psi) for varying durations (10, 20, or 30 min). Following decompression, the lengths of the regenerating cilia were recorded and compared to controls. The regeneration delay for *Arbacia* embryos was found to be a function of the duration of pressure treatment (Fig. 1). The onset of regeneration was delayed for 10–25 min in excess of the duration of compression. This delay was proportional to the duration of treatment. It was not possible to ascertain from this data whether or not the rate of cilia regeneration was affected by the pressure treatment. Photomicrographs of representative experimental (10,000 psi for 20 min) and control embryos 61 min after deciliation are shown in Figure 2. The cilia in the pressure treated embryo measure 4 μ as compared to the control cilia which are 11 μ. The surface cells of pressurized embryos tend to round up during treatment. When the cilia had regenerated to a length of a few microns, ciliary motion was readily evident.

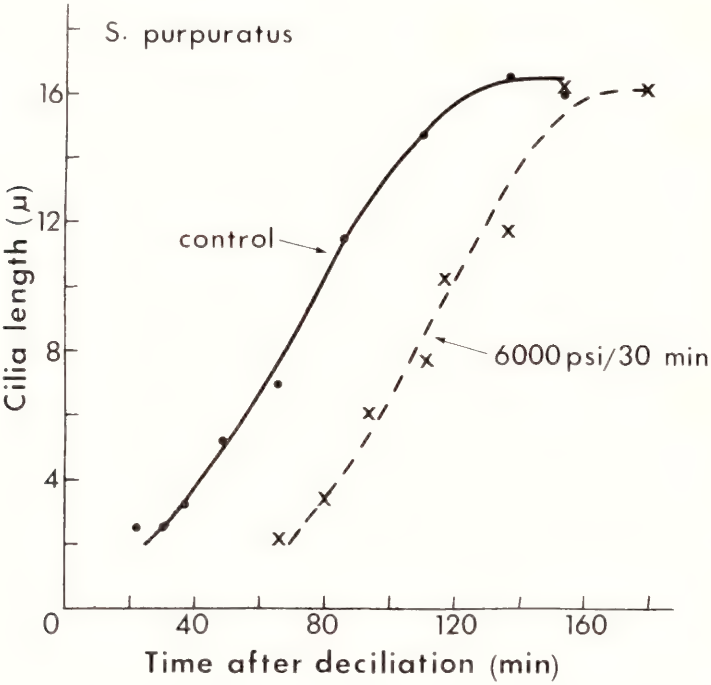

FIGURE 3. Effects of pressure on cilia regeneration in *Strongylocentrotus purpuratus* embryos. Embryos at the hatched blastula stage were subjected to 6000 psi for 30 min immediately after deciliation with hypertonic seawater. Cilia regeneration of control (•——•) and pressure treated cells (x- - - -x) are illustrated.

A similar pattern of regeneration was found for *Strongylocentrotus* swimming blastulae. The regeneration kinetics for a representative series of embryos subjected to 6000 psi for 30 min is shown in Figure 3. The regeneration delay for this pressure-duration treatment was 10 min in excess of treatment. This data suggests that the rate of regeneration was not affected by the pressure treatment.

The effects of various magnitudes of pressure on cilia regeneration was investigated in *S. purpuratus*. The duration was kept constant (30 min) but the magnitude of pressure was varied systematically. In Figure 4 the cilia regeneration curves at three different pressure levels (2000, 4000, and 5000 psi) are shown. The regeneration delay was related to the applied pressure. At lower pressures the cilia were able to regenerate under compression. Short cilia were visible immediately following 30 min compression at 2000 and 4000 psi. At 5000 psi there was no regeneration during compression. As shown in Figure 4, following the release of pressure the regeneration profiles at the three pressure levels were comparable to controls.

DISCUSSION

These studies show that high hydrostatic pressure is capable of profoundly affecting the motility of sea urchin embryos and eventually results in a loss of cilia. In experimentally deciliated embryos, cilia regeneration is blocked at high pressures or retarded at lower pressures for the duration of the treatment.

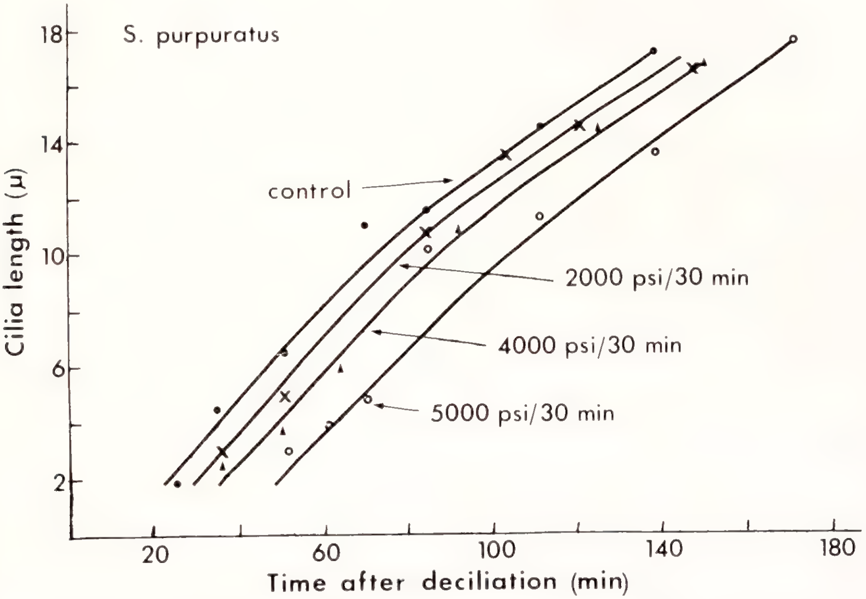

FIGURE 4. Effects of pressure on regeneration in *Strongylocentrotus purpuratus*. Immediately following deciliation by hypertonic seawater, the hatched blastulae were subjected to different magnitudes of pressure (2000, 4000 or 5000 psi) for a standardized duration of 30 min. Cilia length was plotted as a function of time after deciliation.

Relatively few observations have been reported concerning pressure effects on ciliary activity in sea urchin embryos although considerable work has been done on other organisms such as protozoa (Kitching, 1957 and 1970; Kennedy and Zimmerman, 1970). In general, high pressure inhibits the activity of cilia. Tilney and Gibbins (1968, 1969) have reported in *Arbacia* embryos that pressure (6000–7000 psi) results in a slowing or stopping of locomotion and progressive cell disaggregation. However, they did not report any ciliary loss at these pressures for durations up to three hours. This is perhaps explained by the fact that their primary interest was the analysis of fine structure and partial ciliary loss may not have been apparent. They reported that the microtubular elements, the basal body, and rootlet of the cilium were unaffected by pressures of 6000–7000 psi. *Tetrahymena* cilia, however, are quite sensitive to pressure treatment (Kennedy and Zimmerman, 1970). Pressures of 7500 or 10,000 psi induced a degradation of the central ciliary microtubules and a disorganization of longitudinal microtubules after durations of only 2 to 10 min. The lack of observable ultrastructural effect on sea urchin cilia may perhaps reflect a basic difference in ciliary structure from that found in *Tetrahymena*, although this is not readily apparent. The present studies indicate that sea urchin cilia (or associated structures) are affected by pressures in excess of 6000 psi, at least in the region of the base of the cilium, since pressure induces the loss of cilia as apparently intact units. The significance of the bulbous tips observed on detached cilia following pressure treatment is not known, although such tips have been observed in cilia removed by other means (Auclair and Siegel, 1966). In flagellae a bulbous tip can be produced by the rolling up of the axoneme inside the flagellar membrane (Rosenbaum and Child, 1967).

The mechanism by which pressure induces cilia loss is not known. On the basis of the present study it is difficult to speculate since even the precise level of amputation is in doubt. It is possible that the cytoplasmic microtubules near the basal body and/or the cortical plasmagel may be involved in anchoring the cilium since these structures have been shown to be pressure sensitive (Tilney and Gibbins, 1968 and 1969; Marsland, 1970). Recently Blum (1972) has proposed that there is a specialized breaking point in the transitional region which lies between the kinetosome and the ciliary shaft.

It has been convincingly demonstrated (Auclair and Meismer, 1965; Auclair and Siegel, 1966) that initial cilia formation and regeneration do not depend on RNA or protein synthesis in sea urchin embryos. This implies that a considerable pool of the necessary proteins exists in the cells (Auclair and Siegel, 1966). More recent work utilizing pactamycin as a protein synthesis inhibitor indicates that protein synthesis may be necessary for regeneration (Child and Apter, 1969). This delay in regeneration may, however, be due to non-specific effects of the drug since earlier studies had shown that puromycin did not block regeneration although 89% of cellular protein synthesis was inhibited (Auclair and Siegel, 1966). Inhibition of regeneration by pressure, therefore, is probably through interference with assembly processes, possibly the polymerization of the microtubules. It has already been well established that pressure is capable of depolymerizing and preventing the reformation of microtubules in the cytoplasm (Tilney and Gibbins, 1968 and 1969), the mitotic apparatus (Zimmerman and Marsland, 1964) and the axopodia of Heliozoa (Tilney *et al.*, 1966). Although the intact cilium in the sea urchin appears to be insensitive to pressure as regards to depolymerization it is clear from the data that cilia formation is pressure sensitive and that growth rate can be slowed at lower pressures or totally inhibited at higher ones. This may be a result of a shift in a dynamic equilibrium between polymerized and free microtubular subunits. The reason for the excess delay is unknown although it probably reflects reparable cellular damage of some type.

SUMMARY

The effects of hydrostatic pressure on the cilia of sea urchin embryos (*Arbacia*

and *Strongylocentrotus*) were investigated. At a pressure of 10,000 psi the swimming blastula and early gastrula embryos became less active. They lost their translational movement and began to rotate slowly on the bottom of the chamber; in about 10 min all movement stopped and essentially all cilia had fallen from the embryos. At lower pressures and with longer durations the embryos were differentially affected and there was considerable variation in the number of cilia removed from individual embryos. With pressures of 6000 psi for 60 min the majority of the embryos lost more than 50% of their cilia. *Arbacia* embryos were more pressure-sensitive than *Strongylocentrotus* embryos.

Following deciliation with hypertonic seawater, hydrostatic pressure above 5000 psi was found to block regeneration for the duration of the pressure treatment. At 6000 psi and above the regeneration delay was in excess of the duration of pressure treatment; the regeneration delay was directly proportional to the duration of treatment. At pressures lower than 5000 psi sea urchin cilia were able to regenerate under pressure but at a reduced rate relative to controls. Pressure treatment does not affect regeneration rate following decompression.

The results are discussed in terms of the known effects of pressure on cellular systems.

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Vol. 143, No. 2 October, 1972

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

CHEMOTACTIC AND GROWTH RESPONSES OF MARINE BACTERIA TO ALGAL EXTRACELLULAR PRODUCTS

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It has been known for many years that filtrates from axenic algal cultures may be enriched with organic compounds. These materials, including simple amino acids and peptides, sugars, polyalcohols, and occasionally vitamins, enzymes, and toxins, are usually lumped under the term "extracellular products" (Fogg, 1966). Studies using natural populations of phytoplankton have shown that extracellular products are not mere laboratory artifacts, and that, depending upon environmental conditions, they account for 1–20% of the total photoassimilated carbon (Hellebust, 1965; Nalewajko, 1966; Samuel, Shad and Fogg, 1971; Thomas, 1971).

The potential significance of extracellular organic material in marine food chains is extremely interesting. Many authors (Fogg, 1966; Brock, 1966; Alexander, 1971; Whittaker and Feeney, 1971) have suggested that these products may play an important role in marine food chains, especially as potential nutrients for bacteria. However, to our knowledge, there is no direct evidence that this is so—although the ability of bacteria to grow in algal cultures (Vela and Guerra, 1966; Berland, Bianchi and Maestrini, 1969) might be interpreted to support such conclusions.

If, in fact, algal extracellular products are important contributors to bacterial food chains, it would seem possible to construct an aquatic counterpart of the well-known "rhizosphere" of terrestrial ecosystems (Rovira, 1965). A zone may exist, extending outward from an algal cell or colony for an undefined distance, in which bacterial growth is stimulated by extracellular products of the alga. For purposes of discussion in this paper, we will term this region the "phycosphere."

Motile bacteria commonly exhibit chemotaxis to concentration gradients of organic material (Weibull, 1960; Adler, 1969). The ecology of chemotaxis by organotrophic bacteria has not been well studied, but highly species-specific responses to certain carbohydrates, amino acids, and nucleotide bases have been observed (Fogel, Chet and Mitchell, 1971), and certain predatory microorganisms have been shown to be chemotactic to their prey (Chet, Fogel and Mitchell, 1971).

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It is possible that chemotaxis may also be of importance in the establishment of a phycosphere microflora.

In the current studies, we have investigated the validity of the phycosphere concept in a number of ways. We have especially been concerned with the existence and importance of the phycosphere effect during various stages of algal cell growth and death, and the relationship of bacterial chemotaxis to the establishment and maintenance of a phycosphere microflora.

Materials and Methods

Axenic cultures of marine algae were kindly supplied by R. R. L. Guillard, Woods Hole Oceanographic Institution. The following were used in the studies reported here: *Skeletonema costatum* (clone SKEL), *Cyclotella nana* (clone 3H), *Dunaliella tertiolecta* (clone DUN), *Isochrysis galbana* (clone ISO).

The algae were maintained on culture medium F/2A, a slight modification of sea water enrichment medium F/2 (Guilliard and Ryther, 1962), containing 1000 μM $NaNO_3$ and 50 μM K_2HPO_4 per liter. All cultures were grown in 50-ml batches in 125-ml Erlenmeyer flasks at 16° C with 900 foot-candles fluorescent illumination supplemented by four 25 w incandescent bulbs; lights were programmed to an 18-hr-on, 6-hr-off cycle. Cell counts were made using a brightline hemocytometer (American Optical Co., #1492). Cultures were routinely transferred every 10 days.

Algal culture filtrates were obtained by centrifuging aliquots 5 min at 1000 rpm in a table-top clinical centrifuge (International Equipment Co., model CL), followed by sterile filtration through 0.45-μm membrane filters (Millipore HA) which had been pre-washed with a total volume of 10 ml synthetic sea water. Microscopic examination of cells following centrifugation and filtration failed to reveal any detectable cell damage not already in the cultures being sampled. In order to cancel the effects of any organic material introduced into the filtrates from the Millipore filters, F/2A, synthetic sea water, and natural sea water were similarly filtered when used as controls in chemotaxis experiments. All filtrates were stored frozen in screw-capped vials until just before use.

Bacteria were cultured on medium ISOL having the following ingredients per liter: 5.0 g peptone (Difco), 1.0 g yeast extract (Difco), 0.001 g K_2HPO_4 filtered or synthetic sea water to volume. When used for plating, this medium was solidified with 1.5% agar (Difco).

The synthetic sea water medium (SSW) used in these experiments had the following composition per liter: 1.13 g $CaCl_2$, 7.0 g $MgSO_4$, 5.3 g $MgCl_2 \cdot 6H_2O$, 0.72 g KCl, 25 g NaCl, distilled water to volume.

The composition of SSW was chosen to approximate the composition of natural sea water in terms of the most abundant salts. It also satisfies the major ionic requirements of most marine bacteria (MacLeod, 1965). The general suitability of this synthetic sea water is reflected in the fact that all bacterial isolates obtained during these studies could readily be grown on ISOL in which SSW had been substituted for natural sea water. Of course, all bacterial media are selective in one way or another, and the media reported here are no exceptions. Problems introduced by our "selective" media were not evaluated, but they are believed to be minor.

Bacterial isolates were obtained from enrichments of aseptically-obtained sea

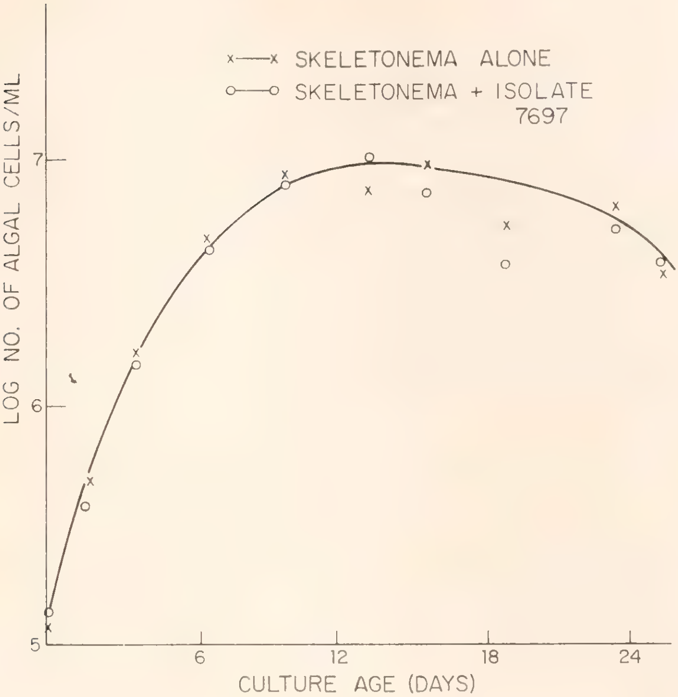

FIGURE 1. Growth of *Skeletonema costatum* in batch culture in the presence and absence of the bacterial isolate 7697. No effect of the bacterium on algal growth was evident in these experiments.

water samples taken from Vineyard Sound near Woods Hole, Massachusetts. The enrichment, begun within 2 hrs after obtaining a sample, consisted of 5 ml of sample + 50 ml of ISOL in 125-ml Erlenmeyer flasks. These were incubated with shaking at room temperature (approximately 27° C) for 18–36 hr before use. These techniques essentially selected for those bacteria able to grow rapidly on a rather rich complex medium. There was no particular selection for specific nutritional types other than for bacteria able to grow aerobically. A more specific technique used to obtain bacteria responding to algal culture filtrates is reported later in this paper. Overnight enrichments always contained a large number of highly motile bacteria.

Chemotactic assays of bacteria, whether obtained directly from mixed enrichments or from pure isolates, were complicated by a variety of behavioral nuances, including the rapid settling of some bacteria onto surfaces and general loss of response upon repeated subculturing. These problems were not completely overcome, but the following procedure yielded the most reliable bacterial preparations: 50-ml cultures were grown on ISOL overnight at room temperature with shaking: 3–6 hr before an experiment, a 2-ml aliquot was inoculated into a second 50 ml of ISOL. After the culture became turbid, the cells were harvested by centrifugation 10 min in a clinical table-top centrifuge (International Equipment Co., model CL) at 2800 rpm; although the supernatant remained slightly turbid, an appreciable pellet was always obtained. The supernatant was drawn off and the cells resuspended in 5 ml sterile SSW. After a second centrifugation, the cells were resuspended again in sterile SSW to a concentration of 10^6–10^7 per ml. Microscopic examination of such preparations showed that 10–90% of the bacteria were motile; only preparations having a high degree of motility were utilized in experiments.

Bacterial chemotaxis was assayed using the method of Adler (1969). Agar plugs were used to close the 1 μl capillaries. Chemotaxis experiments were run at room temperature for 15 to 45 min. During this time bacterial motility and behavior was monitored visually in selected preparations and controls with a phase contrast microscope. At the end of an experiment the contents of each capillary tube were diluted into 10 ml of sterile SSW and bacterial counts made by plating aliquots on solidified ISOL. Chemotaxis experiments were run in duplicate or triplicate.

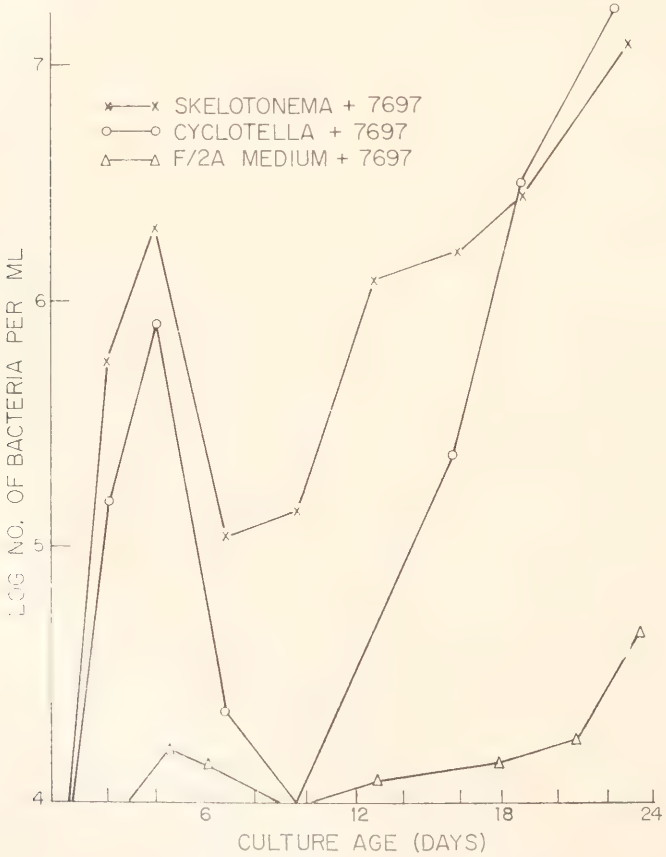

FIGURE 2. Growth of the bacterial isolate 7697 in the presence and absence of two algae, *Skeletonema costatum* and *Cyclotella nana*. The *Skeletonema* culture is the same one depicted in Figure 1. Bacteria were inoculated into these cultures at 0.5×10^4 per ml.

Results

In the first series of experiments, we examined the growth of mixtures of algae and bacteria. These experiments were designed to test for production of either anti-bacterial toxins or bacterial stimulants by the algae. Pure cultures of motile bacterial isolates were washed free of growth medium by centrifugation and resuspension in sterile SSW, then inoculated into freshly transferred axenic algal cultures. Controls consisted of separate algal and bacterial cultures in F/2A medium inoculated at the same time and incubated under the same conditions. The only major carbon source for the bacteria consisted of extracellular material produced by the algae.

Typical results from these experiments are shown in Figures 1 and 2. The two bacterial isolates tested had no discernible effect on algal growth. However, viable cell counts showed that the bacteria were strongly affected by the presence of algal cells. There was typically an initial burst of bacterial growth during the first week of culture. This was followed by a period of either no increase or a significant decrease in viable cell count, approximately coinciding with the transition period between logarithmic and stationary algal growth. Invariably a marked increase in viable bacterial cells was observed as the cultures aged further.

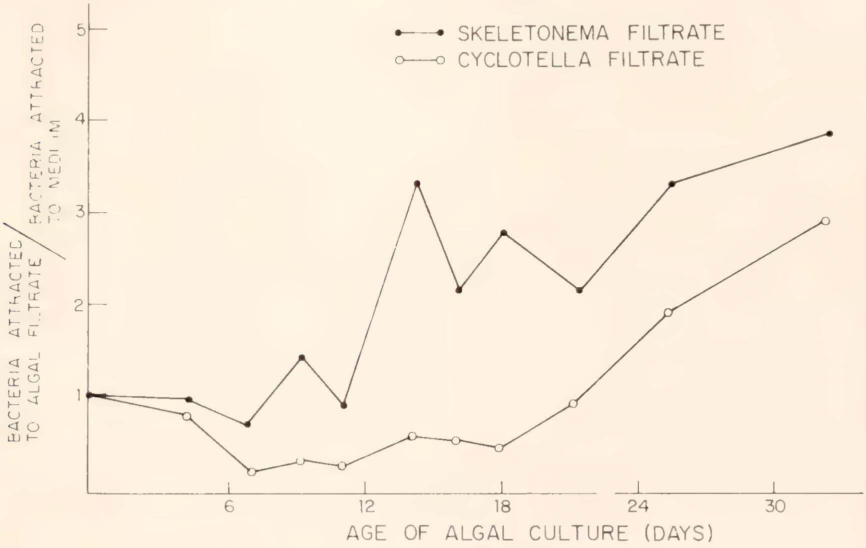

FIGURE 3. Chemotactic response of bacterial isolate 7697 to filtrates from algal cultures of increasing age. Values on the ordinate are given as the ratio of the number of cells in capillary tubes containing algal culture filtrate to the number in tubes containing F/2A medium. Experiments lasted 45 min. at room temperature.

When compared with the experimental flasks, bacterial growth in the controls was insignificant. The bacterial concentration was always several orders of magnitude less. A slight increase in viable cells during the first week probably represents growth on the organic material present in the natural sea water base of F/2A medium; the gradual increase with age can be accounted for by evaporation of the medium in the flasks.

It is evident that the bacteria tested are able to coexist in culture with these algae. The data suggest that most of these bacteria depend on degradation products of the algal cells. A small residual population of bacteria existed in a viable state throughout the 30-day period tested, even in control flasks.

In order to study the possible ecological role of these materials further, an assay utilizing bacterial chemotaxis was developed and employed in a series of experiments with intentions similar to those reported above—*i.e.*, the determination of the effect of algal culture age on bacterial response. Controls for these experiments consisted of capillary tubes containing filtered F/2A of the same age as the algal culture being tested.

The results of one such experiment are shown in Figure 3. The bacterium was isolate 7697, one of those used in the previous growth experiments. The chemotaxis pattern obtained is typical and can profitably be compared with the growth data of Figure 2. Filtrates from young algal cultures did not attract the bacterium,

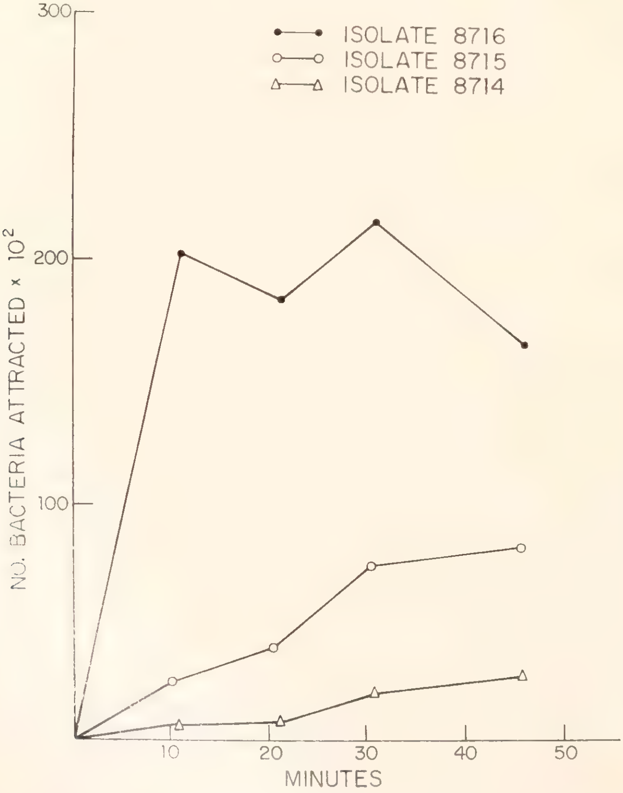

FIGURE 4. Attraction of bacteria from enriched Vineyard Sound water to filtrate from 30-day *Skeletonema* culture. These bacteria, from the same water sample, could be readily differentiated on plates on the basis of colony morphology and color. No other bacteria appeared in significant numbers in this experiment.

but chemotaxis increased markedly when filtrates from older cultures were used as attractants.

Taken together, the data presented in Figures 1–3 strongly suggest the stimulation of a "phycosphere effect" by the algae. However, the data obtained indicate that algal excretions are important only after algae have ceased rapid growth and commenced decomposition.

A series of experiments were initiated to examine the ability of algal culture filtrates to select for specific bacteria from a mixed population. This process is critical in the construction of a phycosphere effect mediated by algal extracellular products. We utilized filtrates from 30-day algal cultures as attractants. These were selected because of their maximum attractiveness to bacteria in previous experiments. Adequate controls were difficult to construct, but Millipore filtered algal medium F/2A of the same age was used as the best compromise. Because bacterial populations in natural sea water are too low to assay quantitatively by this technique, overnight enrichments of whole water samples were tested. No attempt was made to isolate "dominant" bacteria from the water samples. This is justifiable, since any true phycosphere would also be an enrichment bearing little relation to the dominant bacteria outside the zone of influence (Rovira, 1965).

Table I

Attraction of bacterial isolates to filtrates from 30-day algal cultures and to a peptone solution. Data are expressed as for the ordinate in Figure 3; 30-day old uninoculated F/2A medium was used in control tubes. Bacterial response is statistically significant (P = 0.05) if the ratio is 2.0 or greater

Algal culture filtrates	Bacterial clone			
	8712	8714	8715	8716
	No. of bacteria attracted/No. attracted to control medium			
Skeletonema	4.2	3.2	2.9	65
Cyclotella	3.4	5.2	—	95
Dunaliella	3.3	3.4	2.1	122
Isochrysis	0.3	3.1	0.8	51
0.5% peptone	7.9	3.3	6.7	46

Figure 4 shows the results of one such experiment using a mixed bacterial enrichment from Vineyard Sound. The three bacterial types indicated could readily be distinguished on the basis of colony appearance on the counting plates. Other bacteria may have been present but were not seen at the dilutions counted. These data indicate that algal culture filtrates are indeed capable of eliciting chemotactic responses from a non-specific enrichment, and presumably from indigenous bacteria in the water column, with the degree of response differing between bacterial types. Such observations can be exploited in the laboratory for the purpose of obtaining bacteria that respond to specific algal species. Because these experiments utilized a mixed bacterial system, however, caution must be used in labelling bacteria as "strong" or "weak" in their response, as the presence of other bacteria may lead to undefinable interactions.

During the visual monitoring of this particular experiment, variations in bacterial behavior could be readily discerned and later correlated with the specific isolates obtained. The *Spirillum*, isolated as clone 8716, entered the capillary tube extremely rapidly and within 15 min could be found along the entire length. Such behavior is characteristic of spirilla and is often utilized in their isolation (Veldkamp, 1970). The small pseudomonad, isolated as clone 8715, entered less rapidly but by the end of the experiment it had formed a band of high concentration just inside the mouth of the tube. Isolate 8714 was at too low a concentration in the tube to be studied visually.

After their isolation, bacterial strains were tested separately for their ability to respond to 30-day algal culture filtrates and compared with their response to 0.5% peptone, a rather rich organic attractant (Table I). In general, the isolates responded to the culture filtrates as well as, or better than, peptone, with the exception of *Isochrysis* filtrate. The best response was shown by isolate 8716, paralleling its behavior in the mixed enrichment. Despite the possibility of interspecific bacterial interactions in the mixed enrchments, the data of Table I confirm that the bacterial responses are independent of other bacteria.

From the above data it can be concluded that specific bacteria may be selected from a non-specific mixture by algal extracellular products. This selection would be mediated by the chemotactic responses of the bacteria. Subsequent experiments were designed to evaluate this possibility to see if the laboratory studies could be extrapolated into natural systems.

TABLE II

Survey of bacterial chemotactic responses to chemicals identified in filtrates from marine algal cultures. Data expressed as for ordinate in Figure 3, with SSW serving as a control The response is significant ($P = 0.05$) if the ratio is greater than 2.0. All chemicals were tested at a concentration of 10^{-2} M in SSW

Attractant	Bacterial clone			
	8712	8714	8715	8716
	No. of bacteria attracted/no. attracted to control mediu			
Amino acids:				
alanine	4.3	1.3	7.2	24
valine	5.3	0.7	5.3	24
proline	1.7	1.3	4.6	9.5
lysine	2.1	0.4	3.4	146
arginine	8.9	1.4	5.6	87
methionine	8.6	2.5	4.1	—
glutamic acid	0.2	0.0	0.1	0.0
aspartic acid	0.0	0.0	0.0	0.0
Polyalcohols:				
mannitol	0.8	1.3	1.8	43
glycerol		1.5	1.5	1.0
Sugars:				
glucose	1.1	1.8	1.0	1.7
sucrose	1.3	4.9	2.2	39

The construction of adequate control experiments is difficult because of the labile nature of algal culture media. These can change their properties with age whether algae are present or not (Provasoli, McLaughlin and Droop, 1957). We did not detect any significant change in any of our media during thirty days of storage. F/2A was therefore used routinely when testing for chemotaxis to algal culture filtrates. However, SSW, expected to have a lower background concentration of organic material, was employed when testing for chemotaxis to specific chemicals.

Hellebust (1965) reported on the nature of extracellular products from many marine phytoplankters, including those algae studied here. These compounds included simple amino acids, sugars, and polyalcohols; extracellular peptides and polysaccharides were also implicated by the increase in amino acids and monosaccharides after acid hydrolysis of culture filtrates.

If extracellular products are to be implicated as bacterial attractants or related in any other way to the establishment of a phycosphere microflora, the compounds identified by Hellebust (1965) would be expected to be among the active components.

Several of the fresh bacterial isolates shown to be chemotactic to algal culture filtrates were tested for their chemotactic response to Hellebust's (1965) extracellular products. The results (Table II) show that the amino acids elicited the best responses. Curiously, the common metabolite glucose was not a good attractant, though most bacteria responded to sucrose. Glycerol and mannitol, identified in extracellular products by Hellebust (1965), were generally not atractive. The response to glutamic and aspartic acids was low, due to inhibition of bacterial motility around the mouth of the capillary tube. When these compounds were tested dissolved in sea water, there was no chemotactic response although motility was not inhibited.

The absolute concentrations of specific compounds among the extracellular products of marine algal cultures is technically difficult to determine because of problems associated with desalting the medium prior to concentrating the organic material. Some preliminary experiments in this laboratory, studying the extracellular production by the marine alga *Chlorella* sp. (Woods Hole clone 580) indicate concentrations of 10^{-8}–10^{-6} M for amino acids and sugars found in the filtrates of log phase cultures.

Experiments to determine the threshold for bacterial chemotactic response were constructed to compare with such information. These experiments consisted of chemotaxis assays of single bacterial preparations using increasing tenfold dilutions of selected organic compounds in SSW. In almost all cases, the values were found to lie between attractant concentrations of 10^{-6}–10^{-5} M. This range agrees well with threshold concentrations found for chemotactic responses of *Escherichia coli* to monosaccharides as reported by Adler (1969). Considering the very low concentrations of organic material found in algal culture material and likely to be found in natural waters, these thresholds are surprisingly high.

DISCUSSION

The construction of a theory to account for the development of a phycosphere is dependent on two criteria. There must be a source of enrichment for the microbial population in proximity to the algae. The microflora must respond to the algal products by being attracted and/or growing in this region. The data presented will be discussed from the point of view of these criteria.

The chemical nature of algal extracellular products renders them likely sources of microbial nutrients. As they may constitute a significant portion of primary production, such compounds are indeed of potential significance in microbial food chains. There is considerable confusion in the literature, however, as to the source of these compounds. Short-term experiments such as the ones of Fogg, Nalewajko and Watt (1965) and Watt and Fogg (1966) strongly suggest that the materials may be released as products of cell metabolism, a process sometimes termed "excretion." On the other hand, long-term experiments lasting several days such as those of Marker (1965) have shown that the increase in soluble organic carbon in algal culture filtrates might readily be attributed to cell lysis, on the order of 1 in 100–1000 cells daily. Accurate determination of cell lysis by counting techniques has so far not proved technically feasible.

This difficulty in determining the actual source of extracellular organic material under natural conditions complicates ecological interpretation of the effect of this material on bacterial populations. In our study (Figs. 1 and 2) we were able to demonstrate that bacteria were indeed able to grow in algal cultures with no additional carbon source—an observation known to all workers who routinely isolate algal cultures and by no means a novel one (Berland, Bianchi and Maestrini, 1969).

The data contain two additional important observations, however. There was no discernable predation of bacteria on the algae. This reinforces the conclusion that the material on which the bacteria were growing was indeed extracellular. In addition, bacterial growth was maximal during the declining stage of the algal growth curve, when algal cell lysis was evident. It was often possible to observe the presence of bacterial aggregates around clumps of lysed algal cells.

The observations shown in Figure 2 also include an increase in bacterial concentration during the early stage of algal growth. The source of organic material for this increase has not been determined, although it probably was extracellular. It appears, however, that algal extracellular products may have the greatest impact on the bacterial community only during the latter stages of a phytoplankton bloom, when algal cell lysis is highest.

The second criterion for the establishment of a phycosphere, bacterial response, was studied in more depth. The data show conclusively that bacteria are capable of growing in algal cultures. The behavioral response, in this case chemotaxis, was studied from the belief that if a phycosphere were ecologically significant, motile bacteria might be attracted to this region before commencing growth on the organic material.

Our data indicate that marine bacteria are chemotactic to algal culture filtrates. This response was invariably highest to filtrates from old algal cultures (Fig. 3), implying that the release of extracellular carbon is most important ecologically during the later stages of a plankton bloom. There was no significant chemotactic response to filtrates from younger cultures, even though such cultures supported bacterial growth.

The threshold concentrations of some of the compounds eliciting bacterial chemotaxis were found to lie generally in the range 10^{-5}–10^{-4} M. Nearshore waters and estuaries usually average 10^{-6}–10^{-5} M for carbohydrate and 10^{-8}–10^{-7} M for specific amino acids (Wagner, 1969). Bacteria were not attracted to natural sea water (compared against SSW) in our experiments. Utilizing bottles incubated *in situ* in freshwater lakes, Fogg and Watt (1965) found that *Chlorella pyrenoidosa* produced a maximum concentration of extracellular glycollic acid of 1.5 mg/l, or about 10^{-5} M. In our own laboratory, the concentrations of extracellular amino acids and sugars in algal cultures are at best an order of magnitude less during logarithmic growth. In both cases, such experiments have utilized closed systems in which the extra cellular products could accumulate unnaturally. Thus, relative to the general concentrations of organics found in nature, the thresholds for bacterial chemotaxis are very high.

These data do not support the second criterion for a phycosphere as far as rapidly growing planktonic algae are concerned. No bacterial response to young algal culture filtrates was observed. While the possibility of bacterial inhibition cannot be eliminated completely, observations of bacterial behavior during such experiments failed to reveal any noticeable inhibition of bacterial motility or general activity. The interpretation most consistent with these data is that the concentrations of extracellular compounds in filtrates from young algal cultures simply were not above the required thresholds for bacterial attraction. In the filtrates from older algal cultures, it would appear that the second criterion for the creation of a phycosphere is met, at least in terms of bactrial chemotactic response to these filtrates.

Bacterial chemotaxis probably serves to keep a bacterial cell near a source of organic material once it has arrived there by chance. A decomposing algal cell thus serves as a bacterial sink. The shock reaction behavior does not aid a bacterium in locating such a sink, but keeps it there once the bacterium gets close enough to respond to the chemical gradient. This effect may be further enhanced by the tendency of motile bacteria to settle onto nearby surfaces rapidly after they begin exhibiting shock reactions in response to a supra-threshold concentration of chemicals. This kind of behavior is very similar to that observed in photosynthetic bacteria responding to a restricted zone of illumination (Pfennig, 1967). Such behavior would help explain the ability of marine bacteria to exist in what is otherwise—in terms of dissolved organic material—a nutritionally poor environment (Jannasch, 1967).

The notion that algal extracellular products must be highly significant in bacterial food chains is far too general to be of much predictive use. The data presented here indicate that a more narrow definition of "extracellular products" is in order, as the bacterial growth and chemotactic response varies greatly with the age of algal cultures and these are, in fact, greatest when algal cells are lysing in old cultures. The role of extracellular compounds released by rapidly growing algae remains to be evaluated, but the general statement that they have a great significance in microbial ecology is totally unwarranted unless qualified by considering highly specific classes of compounds such as antibiotics, vitamins, toxins, *etc.*

We have found that the term "phycosphere" can be a useful one in discussing and evaluating algal-bacterial interrelationships. The phycosphere effect would be expected to be greatest during algal bloom decomposition. This effect is mediated in part by bacterial chemotaxis to organic material released by the lysing algal cells, which serves to keep bacteria in proximity to the cells until most of the available organic material has been utilized. It would appear that the phycosphere is a region of interactions that have only begun to be evaluated.

We thank Miss J. Lang for excellent technical assistance during portions of this work. The research was supported in part by contract #N00014–67–0298–0026 between Harvard University and the Office of Naval Research.

Summary

1. The possibility that planktonic algae possess a "phycosphere," a zone surrounding them created by the production of extracellular products which may serve as bacterial nutrients, is examined.

2. Bacterial growth in algal cultures to which no additional organic material is added is greatest only as the cultures age and algal cell lysis becomes obvious.

3. Marine bacterial isolates are chemotactic to filtrates from algal cultures, but the response is significant only to filtrates from old cultures, again where cell lysis is evident.

4. Specific compounds known to occur as algal extracellular products attract bacteria, but the threshold concentrations for attraction are unexpectedly high when compared with the generally very low concentrations of organic compounds in natural sea water.

5. The validity of the phycosphere concept and its potential importance to marine microorganisms is discussed.

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RESPONSES OF *CHAETOPTERUS VARIOPEDATUS* TO OSMOTIC STRESS, WITH A DISCUSSION OF THE MECHANISM OF ISOOSMOTIC VOLUME–REGULATION

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Detailed studies on salt and water balance in polychaetes have been primarily directed toward free-living intertidal and estuarine species (see Olgesby, 1969a and 1969b, for extensive reviews). Less information is available on sedentary tube-dwelling worms particularly those which appear to be stenohaline. One such species, *Chaetopterus variopedatus*, occurs widely along coastal areas in the intertidal to subtidal zones. Although it is usually found in regions of stable high salinity, it has been reported from areas where the salt concentration reaches as low as 20‰ (Gosner, 1971). In addition, *Chaetopterus* is known to occur intertidally in areas of heavy seasonal rainfall which may be presumed to produce some dilution of the interstitial seawater. It has been pointed out, however, that the actual salinities immediately surrounding burrowing or tube-dwelling organisms may be considerably different than a cursory sampling of the surrounding water would suggest (Gunter, 1961; Oglesby, 1969b). It is of interest in this connection that Garrey (1905) could find no evidence that *Chaetopterus* could regulate its salt or water content when subjected to salinities below that of approximately 31‰. The present investigation was undertaken, therefore, to examine in greater detail the responses of *Chaetopterus* to osmotic stress.

MATERIALS AND METHODS

Source and maintenance of animals

Specimens of *Chaetopterus* were obtained from Pacific Bio-Marine Supply Co., Venice, California. In the laboratory, the animals (within their natural tubes) were maintained at 16° C in aerated recirculating aquaria containing 50 gallons of artificial seawater ("Instant Ocean," Aquarium Systems, Inc., Cleveland, Ohio). Worms were fed *ad libitum* daily with "Fryfare" extra fine fish food (Wardley Products Company, Inc., Long Island City, New York). Animals were acclimated for a minimum of 7 days under these conditions before any experiments were performed.

Preparation of test solutions

Hypoosmotic solutions were prepared by diluting the artificial seawater in which the worms had been maintained with an appropriate amount of distilled water.

Weight determinations

For the determination of wet weights, the animals were blotted dry with absorbent paper towels and weighed in air (at 16° C) to the nearest milligram on a torsion balance (The Torsion Balance Co., Clifton, New Jersey).

Collection of coelomic fluid samples

Prior to removal of coelomic fluid, the animals were thoroughly blotted with absorbent paper towels. With the aid of a binocular dissecting microscope, the tip of a 200 μl capillary tube, drawn out to a fine point, was inserted through the integument into the large coelomic cavity of one (or more) of the "fan" segments. Fluid was then drawn into the capillary with the aid of a mouth suction-tube. Depending on the size of the animal, from 50 to 150 μl of fluid could be obtained from a single segment in this fashion. A final sample from each worm (in excess of 125 μl) was obtained by pooling the coelomic fluid from the three fan segments, as needed. The capillary tubes were flame-sealed and stored at $-20°$ C. Since coelomic fluid from these segments was virtually devoid of cells, the samples were not centrifuged prior to making determinations.

Determination of osmotic pressure, Na^+ and Cl^-

Osmotic pressure was measured with an Advanced High Precision freezing-point osmometer (Advanced Instruments, Inc., Newton Highlands, Massachusetts) in the following manner. Duplicate 50 μl samples of coelomic fluid were diluted to 200 μl with glass-distilled water and placed in sample holders (0.2 ml) for determination. The final results were calculated by comparison to a standard curve made from solutions of known osmolarity (standard solutions from Advanced Instruments, Inc.) with correction for electrolyte activity change caused by dilution.

For sodium determinations, duplicate 5 μl samples of coelomic fluid were diluted to 5.00 ml with glass-distilled water. Flame photometry was carried out using a Coleman Jr. II spectrophotometer with flame attachment, and the results compared to a standard curve made from solutions of known sodium concentration.

For chloride determinations, duplicate 5 μl samples of coelomic fluid were diluted to 50 μl with glass-distilled water. Titrimetric determinations were made using an Oxford automatic titrator (Oxford Laboratories, San Mateo, California) with acid mercuric nitrate in the presence of s-diphenylcarbazone as an endpoint indicator. Results were compared to standard solutions of known chloride concentration.

RESULTS

Long-term adaptation to hypoosmotic seawater

To examine the response to long-term, gradual lowering of salinity in the external medium, 50 worms (in their natural tubes) were allowed to acclimate for one week in artificial seawater of 1090 milliosmoles concentration. The health of the worms was estimated by cutting off one of the tips of the "U"-shaped tubes and observing whether or not the tip was repaired. Rapid (overnight) repair of its tube house was taken as a sign that a worm was in good condition. Five worms were removed from their tubes and coelomic fluid and medium samples taken. The salinity in the acclimation aquarium was then gradually lowered at a rate of approximately 3% per day by addition of calculated amounts (volumetric) of distilled water. At varying intervals, groups of six worms in their tubes were transferred to other aquaria and kept for seven days at the lowered salinity attained. All worms were fed daily. At the end of the week period, worms were removed from their tubes, examined, and coelomic fluid and medium samples taken. The data for total osmolarity, sodium, and chloride concentrations are shown in Figures 1–3.

Over the range studied, it appears that the coelomic fluid of *Chaetopterus* conforms to the external medium with respect to total osmolarity and sodium and chloride concentration. There is no indication of ionic or osmotic regulation for the parameters examined. Only a single animal (out of 10) survived the slow dilution and week-long maintenance at 630 milliosmoles (*i.e.*, 57.8% of the initial value). It would appear then, that approximately 55–60% seawater is the lower limit which *Chaetopterus* can tolerate for moderately extended periods of time. Survivorship and/or regulation was not examined in hyperosmotic solutions exceeding *ca.* 1100 milliosmoles.

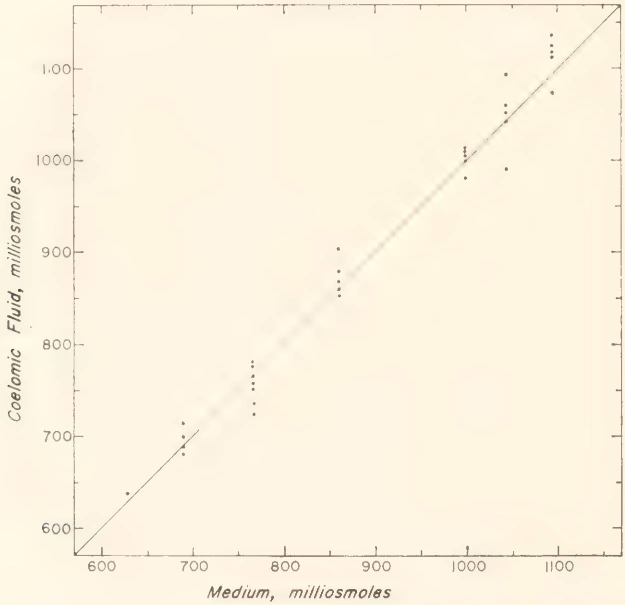


FIGURE 1. The osmotic concentration of *Chaetopterus* coelomic fluid in relation to that of the external medium, all points shown.

Volume changes during osmotic stress

To examine the effects of osmotic stress on water and salt movements, worms were carefully removed from their tubes and placed individually in 4-inch finger

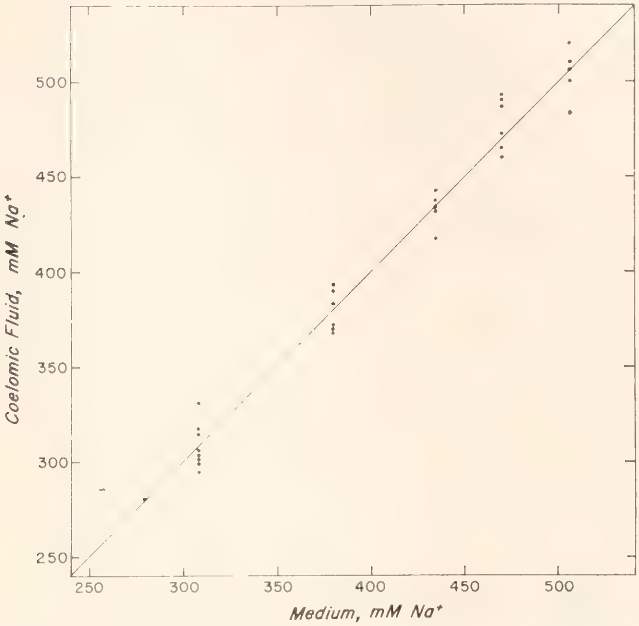


FIGURE 2. The sodium concentration of *Chaetopterus* coelomic fluid in relation to that of the external medium, all points shown.

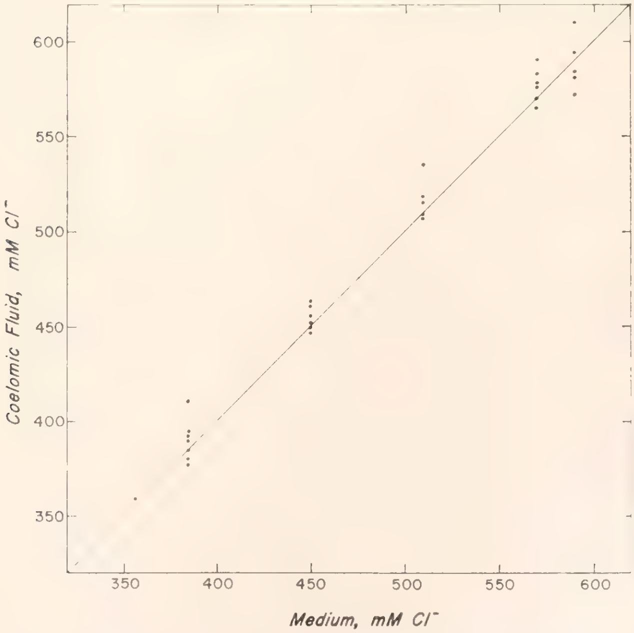


FIGURE 3. The chloride concentration of *Chaetopterus* coelomic fluid in relation to that of the external medium, all points shown.

bowls filled with 200 ml of artificial seawater. They were then carefully examined, and any appearing unhealthy, injured, or incapable of producing the characteristic rhythmic pumping activity were discarded. Stage of maturity and sex were recorded for each of the experimental animals. The worms were distributed by size so that each group (experimentals and control) had a representative size distribution. Initial weights were taken and experimental animals were placed in hypoosmotic test solutions of known salinity. All individuals were then weighed at successive 30-min intervals for a period of six hours, or until they were removed from the experiment because of rupture (group A). After six hours had elapsed, the worms were transferred back to seawater of the initial strength and weight monitored for an additional 2½ hours as before. The data are shown in Figure 4 (as absolute per cent body weight change) and Figure 5 (corrected for controls). Typical variability is indicated by the mean ±1 standard error.

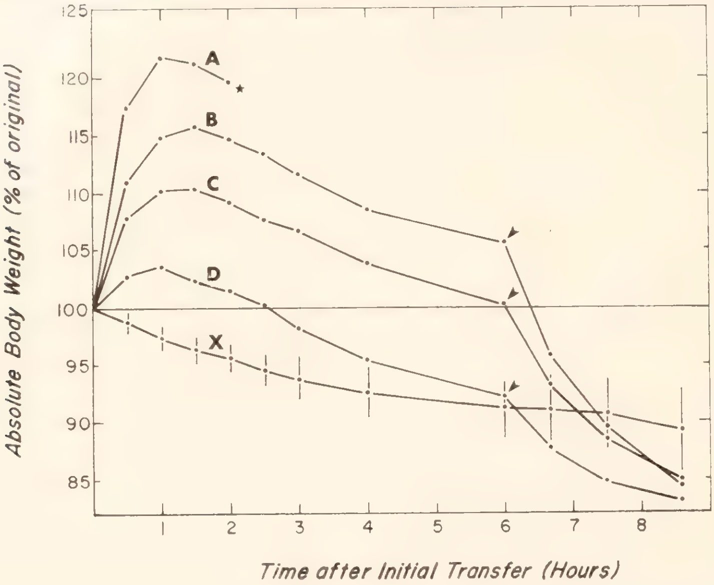


FIGURE 4. Time-course of weight changes in *Chaetopterus* after direct transfers from 901.4 milliosmoles to: (A) 451.4 milliosmoles (n = 5); (B) 681.8 milliosmoles (n = 7); (c) 749.6 milliosmoles (n = 8); (D) 802.8 milliosmoles (n = 10); (X) controls, kept at 901.4 milliosmoles (n = 11). Star on curve (A) indicates all animals had ruptured, and were removed from experiment. Arrows at 6 hours indicate transfer back to initial medium. Vertical bars represent one S.E. above and below the mean, and are indicated for one curve only, for clarity, as representative of the amount of variability encountered in all curves.

The data in Figures 4 and 5 show that: (1) control worms (remaining under acclimated osmotic conditions) slowly lose weight, presumably due to handling; (2) worms subjected to hypoosmotic solutions rapidly gain weight, roughly in proportion to the degree of osmotic stress; (3) weight gain is essentially complete in 1.5 hours, and is followed by a period of both absolute and relative weight loss; and (4) following transfer back to initial salinity, all experimental groups rapidly

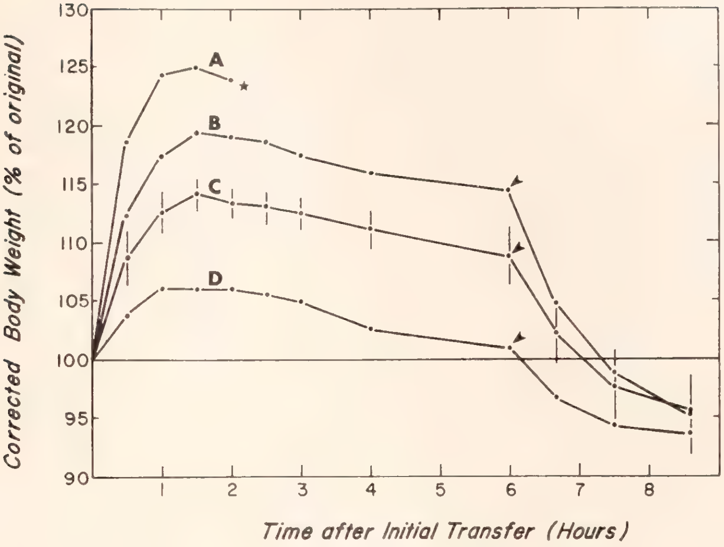


FIGURE 5. As in Figure 4, corrected for controls.

lose weight, ultimately dropping below control group values. No significant differences were found with regard to sex or state of maturity.

To examine further the effects of sudden osmotic stress on weight and coelomic fluid constituents, 30 animals were removed from their tubes, examined, weighed and assigned to control (10) and experimental (20) groups as before. The experimental animals were transferred to a hypoosmotic solution (*ca.* 80% acclimated seawater) at timed intervals. At 1.5 hours elapsed time all animals were weighed, and coelomic fluid samples from five control and five experimental

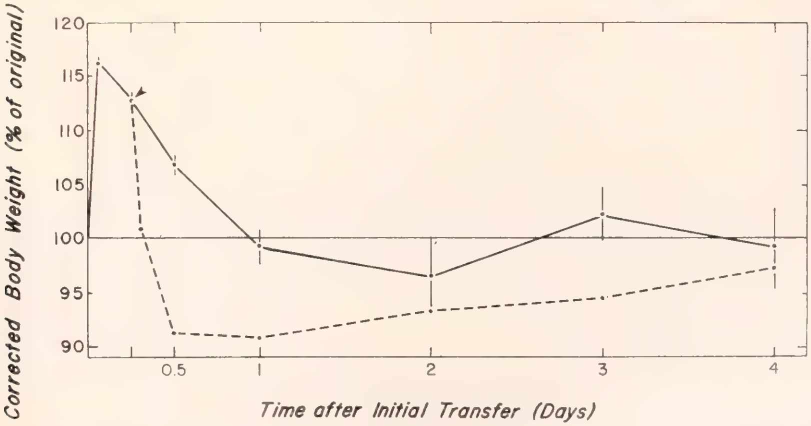


FIGURE 6. Time-course of weight changes (corrected for controls) in *Chaetopterus* after direct transfer from 1100 to 875 milliosmoles. Arrow indicates transfer back to initial medium for one group (broken line). For clarity, ±1 standard error shown for one group only; n = 5 for all points after 6 hours.

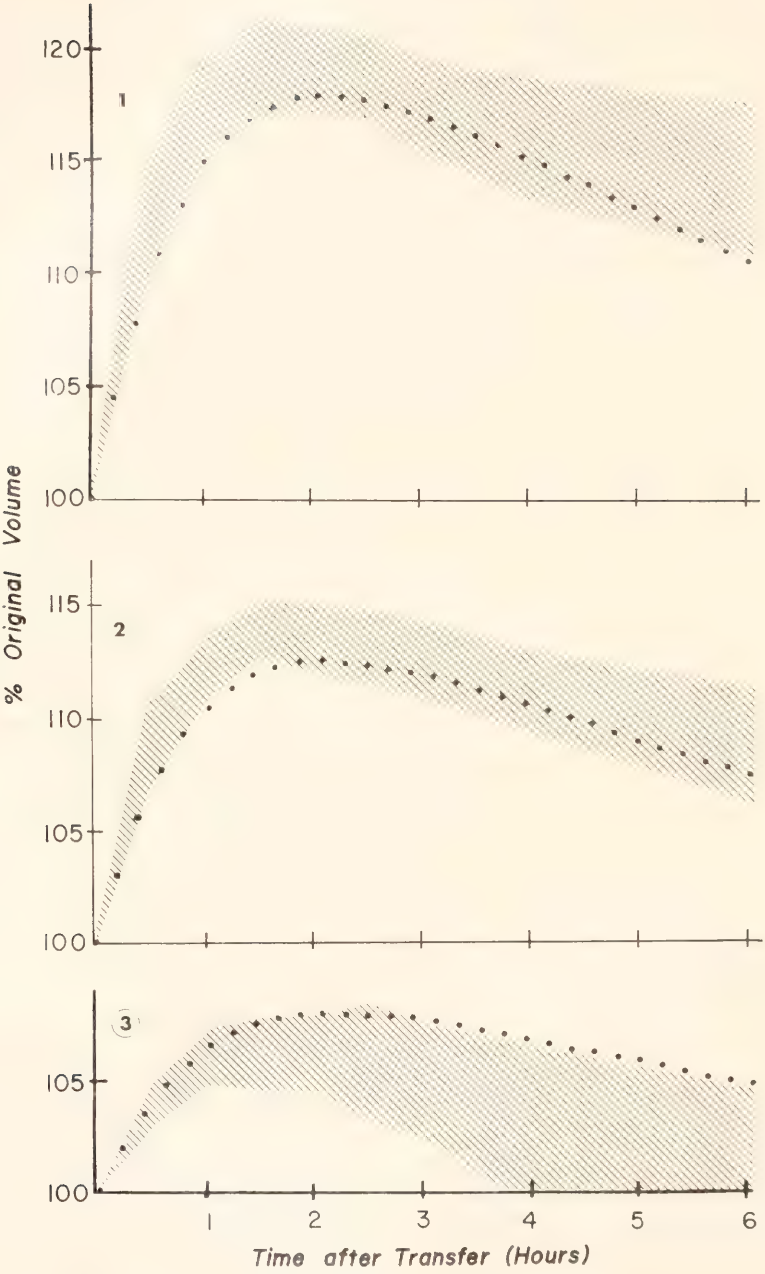


FIGURE 7. Experimental results *versus* behavior predicted from theoretical model. Crosshatch indicates experimental data (mean ±1 standard error); dotted line from computer solution to equations; (1) $\Delta C = 0.76, \beta = -0.30$; (2) $\Delta C = 0.83, \beta = -0.30$; (3) $\Delta C = 0.89, \beta = -0.30$; (4) $\Delta C = 0.76, \beta = -0.29$; (5) $\Delta C = 0.83, \beta = -0.56$; (6) $\Delta C = 0.89, \beta = -0.17$.

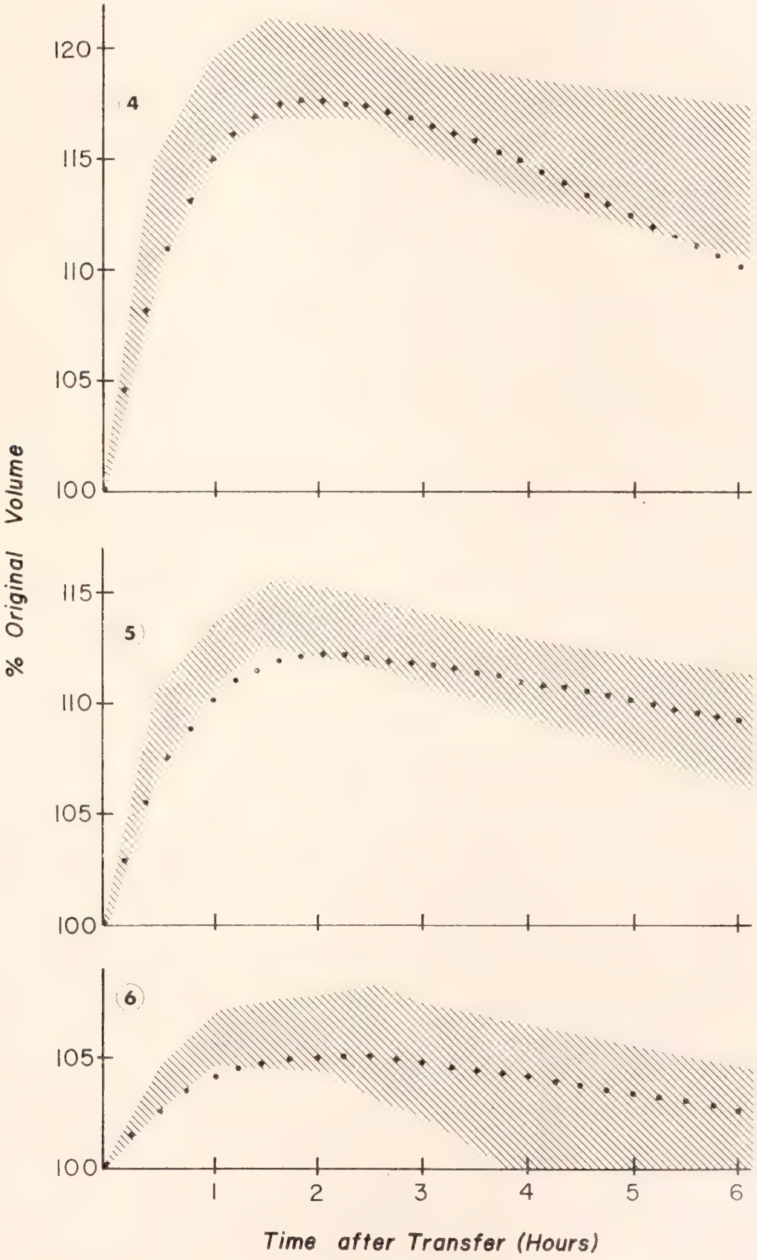


FIGURE 7—(*Continued*)

animals (as well as medium samples) were taken. At six hours all remaining animals were weighed, and ten experimental animals transferred back to initial salinity conditions. One and one-half hours after transfer, five animals were removed from the latter group, and coelomic fluid and medium samples taken. The weight of the remaining 15 animals (five per group) was monitored at regular intervals over a period of four days. The worms were provided with food and substitute "houses" (consisting of glass tubes of appropriate diameter) following the 12th-hour weighing. Clean water of the proper salinity was added daily. Data on the weight changes of the experimental groups (corrected for controls) are shown in Figure 6. The results indicate that animals subjected to sublethal hypoosmotic stress return to approximately initial weight values after about 24 hours (Fig. 6, solid curve). In view of the low number of animals involved and the variability exhibited, no significance is ascribed to the apparent oscillatory behavior of the mean weight around the 100% value. Those animals transferred back to the initial salinity lose weight rapidly, ultimately dropping to *ca.* 90% of their starting value (Fig. 6, broken curve). There follows a slow climb in weight toward the starting values.

Coelomic fluid : medium ratios for osmolarity, sodium and chloride, taken 90 minutes after transfer to new media, are given in Table I. The data show the coelomic fluid to be not significantly different from the medium with respect to osmotic and ionic concentration.

DISCUSSION

Data from the present investigation suggest that *Chaetopterus variopedatus* is an ionic- (Na^+; Cl^-) and osmoconformer at seawater concentrations from 1100 milliosmoles down to a lethal lower limit of approximately 630 milliosmoles. This corresponds to a range of 116%–66% seawater (with "100%" seawater = 950 milliosmoles). These data are consistent with measurements for other marine polychaetes, which indicate that the majority of these annelids are osmoconformers at the higher range of salinities (Oglesby, 1969a). The fact that total osmotic pressure closely parallels the sodium and chloride concentrations indicates that there is only minor contribution (if any) from soluble organic molecules to the total osmotic concentration of the body fluids, even at lowered salinities. Little is known directly about the organic constitutents of *Chaetopterus* body fluids, except that there is no dissolved respiratory pigment (Dales, 1969) and that uric acid occurs at a concentration of 43.4 μMoles/l (Wilber, 1948). It is concluded that the major osmotic characteristics observed result almost exclusively from water and inorganic salt fluxes.

TABLE I.

Coelomic fluid: medium ratios 1.5 hours after transfer to new medium, mean ±1 standard error; n = 5 for all values

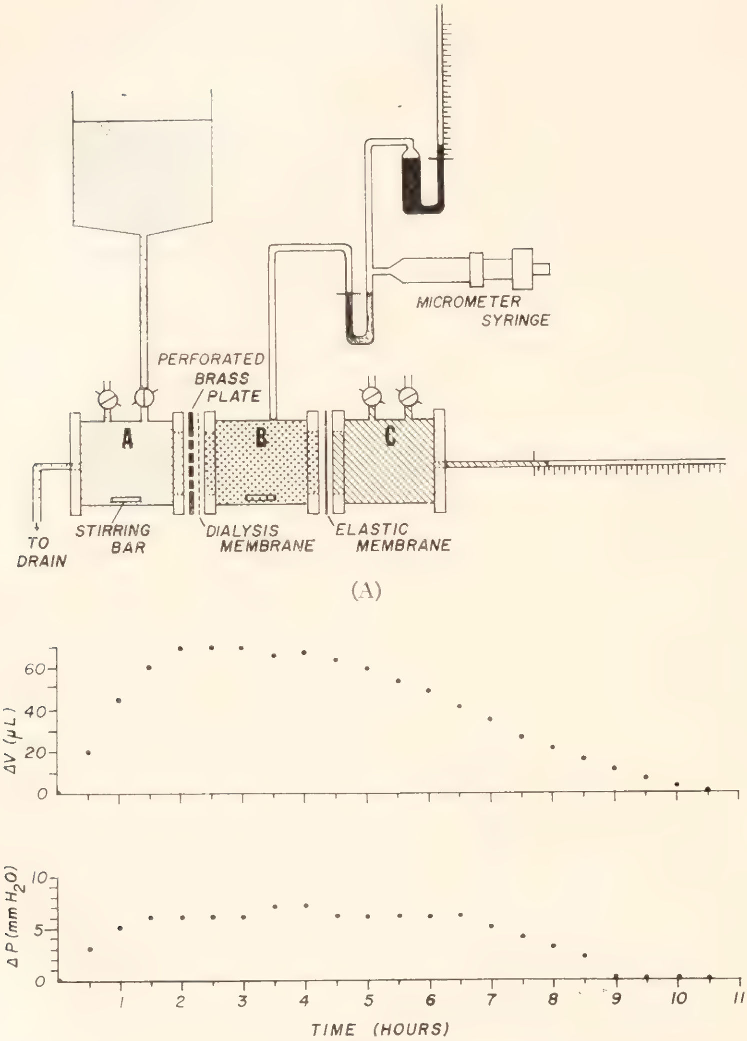
Following transfer:	mOsm (CF:M)	Na^+ (CF:M)	Cl^- (CF:M)
From 1100 to 875 mOsm	1.02 ± 0.029	0.99 ± 0.066	1.06 ± 0.067
From 875 to 1100 mOsm	0.99 ± 0.016	1.01 ± 0.019	0.99 ± 0.011

The rapid increase in weight (volume) of worms directly transferred to hypoosmotic media presumably reflects an osmotically driven influx of water through a readily permeable integument. The volume increase reaches a maximum in 1.5 hours (Figs. 4, 5, and 6) at which time the coelomic fluid has again reached osmotic equilibrium with the medium (Table I). Comparison of initial slopes of weight gain (after initial transfer) with weight loss (after back-transfer) also clearly indicates that, under the same osmotic gradient, fluid enters more rapidly than it leaves. This apparent greater permeability inward than outward has been reported for other species by a number of investigators (Adolph, 1936; Gross, 1954; Jørgensen and Dales, 1957; Oglesby, 1965) although the reason for it is not known.

That some salt efflux has also occurred is indicated from the results of experiments where hypoosmotically stressed worms were returned to initial salinities (Figs. 4, 5, and 6). In every case, worms rapidly lost weight and eventually dropped well below the mean weight of the control groups. Weight loss is essentially complete after $1\frac{1}{2}$–2 hours (Figs. 4, 5, and 6), after which there appears to be a slow climb toward initial values (Fig. 6). Shrinkage to less-than-original volume after transfer back to normal seawater shows that the equilibrium attained in dilute seawater was reached partly by the loss of solutes (Potts and Parry, 1963),

A return to initial weight (volume) values after transfer to media of various salinities is conventionally used as the criterion for judging whether or not a marine animal has the ability to control its body volume (Oglesby, 1969a). Applying this criterion to data on the time-course of weight changes in hypoosmotically stressed *Chaetopterus* (Figs. 4, 5, and 6), one must conclude that *Chatetopterus* possesses volume-regulating ability over most, if not all, of its viable salinity range. Garrey (1905) is apparently the only previous investigator to publish data on the response of *Chaetopterus* to hypoosmotic media. He transferred worms directly from full-strength Woods Hole seawater ($\Delta = -1.82°$ C) to: (1) 50% seawater ($\Delta = -1.02°$ C), and (2) "fresh water" ($\Delta = -0.02°$ C) (Garrey, 1905, Table IV). After periods of six hours in 50% S.W. and three hours in F.W., the worms increased in weight (by an unstated amount) and appeared "swollen." In light of results from the present study, it is evident that Garrey's stress solutions were in the lethal range for the animals. Forced to base his judgment on Garrey's limited data, Oglesby could only conclude that *Chaetopterus* showed "no" ability to regulate its volume during osmotic stress (Oglesby, 1969a, Table X, page 251). The volume-regulating ability of *Chaetopterus* is, in fact, quite comparable in magnitude and rate to some of the better-known volume-regulating nereids, such as *Nereis diversicolor* (Beadle, 1937) and *N. limnicola* (Oglesby, 1968). In contrast to these euryhaline species, however, *Chaetopterus* appears to have no (or minor) salt-regulating capabilities and is unable to tolerate salinities as low as 50% S.W.

The mechanism by which an isoosmotic polychaete, such as *Chaetopterus*, "regulates" its volume after transfer to lower salinities is not known. There are currently two hypotheses to explain the corrective elimination of fluid in such animals. The first suggests that there is an increased efflux of fluid via the nephridia. The motive force driving this efflux may be produced intrinsically (*i.e.*, as a result of increased activity of cilia lining the nephridial tubules) or


PERFORATED
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MEMBRANE
ΔV (μL)
0
20
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60
ΔP (mm H2O)
0
5
10
1
2
3
4
5
6
7
8
9
10
11
TIME (HOURS)

(A)

(B)

FIGURE 8. (A) "Mechanical engine" with properties of model. See text for details. (B) Time-course of volume and pressure changes in chamber B after osmotic gradient established at time 0.

extrinsically (*i.e.*, as a result of increased coelomic hydrostatic pressure caused by contraction of the body-wall musculature) (Beadle, 1937, 1943; Smith, 1970). A second hypothesis suggests that there is active transport of an ion species *outward* through the general integument. There presumably follows the passive diffusion outward of a counter-ion (maintaining electrical neutrality) and the passive diffusion outward of water (maintaining osmotic equilibrium). The total effect is the net transfer of isoosmotic saline from inside to outside (Prosser and

Brown, 1961). Although these proposed mechanisms differ markedly, they are not mutually exclusive. They are similar in presupposing that an expenditure of energy is required to produce the driving force necessary for fluid elimination. At present there is virtually no data to support or refute either of these hypotheses in detail, and even the studies investigating metabolic rates during volume regulation have failed to confirm or deny the existence of an energy requirement (Oglesby, 1969a).

One of the conceptual difficulties inherent in these theories relates to the negative-feedback character of the phenomenon, since data from many sources indicate that in good volume-regulators the amount of fluid efflux is such that the worms are returned rather precisely to their initial volumes (given sufficient time and sublethal stress). With reference to the active transport hypothesis, for example, there are many common systems where changes in salt concentration are known to elicit activity of membrane transport systems which act to correct the perturbation. When the salt concentration again approaches initial values, activity of the transport system is lowered to an appropriate level or stopped. Such monitoring of salt levels may be operative in polychaetes capable of both ion- and osmoregulation at low external salinities. With regard to isoosmotic volume-regulators, however, it would seem that there is a crucial component missing—namely, the internal salt concentration does not return to prestressed values during the following volume decrease. Therefore, a salt concentration "signal" to turn off the corrective effector activity (producing fluid efflux) does not appear to be present. In contrast, the hypothesis that a type of muscular stretch response produces hydrostatic back-pressure at least has the merit of suggesting how a mechanically linked "volumestat" servo-mechanism control might be achieved.

Nevertheless, it appears to us that the principle of parsimony has been all but ignored by the general assumption that the major route of fluid elimination is via the nephridial tubules. The apparent agreement on this point seems particularly puzzling since it is also generally acknowleged that the initial water entry occurs throughout the entire permeable integumental surface. No one has suggested, for example, that the initial rapid increase in volume results primarily from flow of water *inward* through the nephridial tubules. In addition, it is clear that active contraction of the body-wall musculature is not the only potential source of increased internal hydrostatic pressure. Apparently overlooked is the fact that the integumental membrane itself is an elastic or visco-elastic element and therefore must, at the very least, supplement muscular contraction in producing increased hydrostatic pressure.

We have therefore attempted to get some estimate of the magnitude of fluid efflux through the surface membrane of a hypothetical model "worm" in which the sole driving force is generated by stretched elastic or visco-elastic elements in the surface membrane. To do this we draw upon some of the concepts of non-equilibrium thermodynamics and make the following assumptions: (1) for ease in calculation, we model our hypothetical "worm" as a cylinder of uniform cross section of radius r and an unstressed volume V_0, for which we can neglect end-effects; (2) we assume that the bounding ("integumental") membrane of the model worm has *no* gross discontinuities (representing, for example, open metanephridial tubules); (3) we assume that the membrane possesses elastic properties which can be adequately represented by Hooke's Law:

$$\Delta P = -G(r - r_0)$$

where ΔP = the change in pressure across the membrane, G = a constant, and the subscript zero denotes the value in the unstressed state; (4) we assume the membrane to be more permeable to water than to solutes; (5) we assume the membrane is not completely impermeable to solutes; (6) we assume that the permeability inward is equal to, or greater than, the permeability outward; (7) we assume that order-of-magnitude estimates for the values for permeability, change in radius, change in internal salt concentration, response time, *etc.* are identical with those derived from the experiments with *Chaetopterus* reported above; and (8) we assume *no* contribution to the system from contracting elements or active transport processes.

Using the basic equations of Kedem and Katchalsky (1958), the volume and salt fluxes across the membrane are respectively:

$$j = L_p(\Delta P - \sigma RT\Delta C) \tag{1}$$

$$\dot{n} = \omega RT\Delta C + (1 - \sigma)cj \tag{2}$$

where L_p is the filtration coefficient, σ is the reflection coefficient, ω is the solute mobility, R is the gas constant, T is the temperature, and ΔP, $\Delta C = C^0 - C^i$, and $C = \frac{1}{2}(C^0 + C^i)$ are, respectively, the differences in hydrostatic pressure, salt concentration, and the mean salt concentration across the membrane. Since L_p and ω may take on different values for inflow and outflow across the integument, we write

$$\left(\frac{L_p(t)}{\omega(t)}\right) = \left(\frac{L(0)}{\omega(0)}\right)[1 + \beta U(t - t^*)]$$

where β $\left(= \dfrac{L_p^{out} - L_p^{in}}{L_p^{in}}\right)$, is a constant, $U(t)$ is the unit step function, and t^* is the time at which the flow is reversed. Substituting these into (1) and (2), and making the change of variables (Crank, 1956)

$$I(t) = \int_0^t L_p(\tau)d\tau$$

The temporal changes of the radius of the cylinder and the number of moles of salt therein, N^i are given by

$$\frac{dR'}{d\hat{I}} = -\hat{G}R' - [\hat{C} - (1 + N')(1 + R')^{-2}]$$

$$\frac{1}{(1 + R')}\frac{dN'}{d\hat{I}} = \hat{\omega}[\hat{C} - (1 + N')(1 + R')^{-2}] + (1 - \sigma)[\hat{C} + (1 + N')(1 + R')^{-2}]\frac{dR'}{d\hat{I}}$$

where we have introduced the following scaled variables

$$R' = \left(\frac{r}{r_0} - 1\right), \quad N' = \left(\frac{N^i}{N_0{}^i} - 1\right), \quad \hat{C} = \frac{C^0}{C_0{}^i},$$

$$\hat{I} = \frac{I\sigma RTC_0{}^i}{r_0}, \quad \hat{G} = \frac{Gr_0}{\sigma RTC_0{}^i},$$

$$\hat{\omega} = \frac{2\,\omega(0)}{L_p(0)}\,\frac{1}{\sigma C_0{}^i}$$

Finding solutions to the above set of nonlinear equations would be a difficult task. However, observations made on *Chaetopterus* indicate that R′ and N′ do not exceed order 10^{-1}. Therefore, we may linearize the above, which gives

$$\frac{dR'}{d\hat{I}} = -(\hat{G} + 2)R' + N' + (1 - \hat{C}) \tag{3}$$

$$\frac{dN'}{d\hat{I}} = \hat{\omega}[2R' - N'] - (1 - \hat{C})\hat{\omega} + (1 - \sigma)(1 + \hat{C})\,\frac{dR'}{d\hat{I}} \tag{4}$$

Using Laplace transformations, it is a simple matter to find the two characteristic frequencies for (3) and (4) and the solution to our problem. We obtain

$$R' \ (1 - \hat{C}) = e^{\lambda_2 \hat{I}} \sinh \lambda_1 \hat{I} \ \lambda_1 \tag{5}$$

where

$$\lambda_1 = \tfrac{1}{2}\{[\hat{\omega} - (1 + \hat{C})(1 - \sigma) + \hat{G} + 2]^2 - 4\hat{\omega}\hat{G}\}^{\frac{1}{2}} > 0$$

$$\lambda_2 = -\tfrac{1}{2}[\hat{\omega} - (1 + \hat{C})(1 - \sigma) + \hat{G} + 2] < 0.$$

Clearly if $\hat{\omega}\hat{G} > 0$, R′ has the appropriate behavior, since I is a monotone increasing function of time. If we require that both the initial slope and maximum of equation (5) correspond to values obtained from the stress experiments on *Chaetopterus* (Fig. 5), we find

$$L_p(0)\,\frac{\sigma RTC_0{}^i}{r_0} \cong 8 \times 10^{-3}/\text{min.}$$

$$\hat{I} = (8 \times 10^{-3}/\text{min.})t\left[1 + \begin{cases} 0, & t \leq t^* \\ \beta(1 - t^*/t), & t > t^* \end{cases}\right]$$

with λ_1, λ_2 in the range $.156 \leq \lambda_1 \leq .825$, $-1.04 \geq \lambda_2 \geq -1.25$. Figure 7 shows a comparison of the experimentally observed volume changes to those predicted on the basis of the calculations from our model. The curves on graphs 1, 2, and 3 were generated on the assumption that there exists a single L_p and β for the integument, and that our best estimate of these is derived from the mean value from all three experiments. The predicted curves on graphs 4, 5, and 6 were generated on the assumption that the different salinity stresses (and hence the degree of stretching of the membrane) influenced the L_p and β, and that our best estimate of these values is derived from the mean of each individual experiment.

In either case the graphs show remarkably good agreement between the calculated and the observed curves, consideration being given to the approximations in the former and the variability in the latter. We have also examined the case for a symmetrical system ($\beta = 0$), and find that it shows a similar response curve. As β is decreased from zero, the decay time from the state of maximal strain (to zero strain) is increased.

Using our calculations we can make an estimate of the values of the material constants σ, $\hat{G}$, and $\hat{\omega}$ ($\sigma \leq 0.05$, $\hat{G} \simeq 1$, and $\hat{\omega} \simeq 0.5$). Transforming back to unscaled variables, we have $L_p(0) \simeq 0.020$ cm(atm·min)$^{-1}$ $G \simeq 0.52$ atm, $\omega(0) \simeq 8.15 \times 10^{-8}$ moles (atm·min·cm^2)$^{-1}$. Since $R'_{\max}$ is of order 5×10^{-2}, we have $\Delta P_{\max}$ of order 1×10^{-2} atm, which corresponds to a required maximum internal hydrostatic pressure of 7.6 mm of Hg, clearly within the range of measured coelomic pressures of polychaetes (Chapman and Newell, 1947; Prosser and Brown, 1961; Wells, 1945 and 1961). The small σ and $\omega(0)$ obtained are characteristic of a somewhat unselective membrane through which there is little solute flow at constant volume, which is consistent with the characteristics postulated for the integument of *Chaetopterus*.

The observed volume-regulation can be explained by the following mechanism. Initially, solvent flows into the worm as a result of the osmotic pressure difference across the membrane. (At the same time there is likely to be a small loss of salt to the exterior so that correctly the net volume increase equals volume H_2O inward minus volume salt outward. The initial water influx appears to be so rapid, however, that isoosmotic conditions obtain before any great volume of salts is lost and therefore it can be largely neglected.) The kinetic energy of water entry is transferred to the integument as a result of stretching the elastic elements. When the initial osmotic driving force is equalized (*i,e.*. isoosmotic conditions are attained) the energy stored in the distended membrane, via increased hydrostatic pressure, forces both salts and water back out through the membrane into the surrounding bath. The asymmetric nature of volume-regulation curves can be explained by the assumptions that although the fluid initially entering the animal (osmotically) is virtually pure water, the fluid leaving the animal (hydrostatically) must be isoosmotic with the body fluids, since preferential loss of water would set up an osmotic gradient in the opposite direction again. The rate of fluid (volume) loss will be determined by: (1) the hydrostatic pressure, and (2) the permeability of theintegument to both water and salt. Since the integument is less permeable to salts, the rate of efflux of isoosmotic saline will be decreased by this slower-moving component. The homeostatic nature of the mechanism is obvious. When the worm has returned to its original volume, the integument is no longer stretched—the energy stored in the elastic element having been completely dissipated in doing the work of expelling the fluid.

Such a mechanism requires no direct input of metabolic energy to the corrective process. It appeared possible to us, therefore, to construct a "mechanical engine" with (at least most of) the assumed properties of the system. Such a device, made up of 3 contiguous lucite chambers, is shown in Figure 8A. For practical purposes it was necessary to separate the elastic portion of the "integument" from the permeable, and thus the configuration of the mechanical engine was different than that of our theoretical model system (assumption #1, above). However, the properties of the mechanical engine conformed closely to those outlined for our theoretical model in assumptions 2, 3, 4, 5, and 8, with the dialysis membrane being symmetrical ($\beta = 0$)—an alternative possibility in assumption 6. Assumption 7 was clearly impossible to achieve and thus the data are expressed in absolute units, without direct comparison to those derived from *Chaetopterus*. In practice a solute (sucrose) concentration difference of 0.1–0.5 M was initially established across the dialysis membrane, with chamber B (the "worm") hyperosmotic to chamber A (the "external medium"). The changes in pressure and volume in chamber B could be simultaneously monitored, with chamber C serving as a liquid-filled compensation chamber leading to the volume capillary. An example of the type of data obtained from the apparatus is shown in Figure 8B. The general shapes of the curves conform to the predicted behavior and show that such a completely mechanical model system is feasible.

Several comments are in order concerning the proposed mechanical "volumestat" model. First, such a system will only completely return to its initial volume if the membrane acts as a pure Hooke elastic element. We have performed the analysis assuming the integument to have both viscous and elastic properties, and thus to act as a Maxwell element

$$\Delta P = -G\left[(r - r_0) - \lambda \int_0^t e^{-\lambda(t-\tau)}(r - r_0)d\tau\right]$$

Understandably, the resulting curves vary, depending on the proportion of elastic to viscous properties. As G approaches zero (pure viscosity) there is swelling but no return. As λ approaches zero (pure elasticity) there is swelling followed by complete return. When both G and λ are greater than zero, there is swelling and partial return, depending on the specific values of the constants.

Secondly, we are fully cognizant that the integument of *Chaetopterus* is perhaps uniquely thin and distensible among those of the larger polychaetes. Previous histological studies, for example, have shown that *Chaetopterus* has, over large areas of its body, as few as two cell layers separating the coelomic fluid from the outer medium (Bonhomme, 1943; Krekel, 1920; Meissner, 1935; Nicol, 1952; Trojan, 1913). In addition, a recent electron-microscope survey study showed that *Chaetopterus* is exceptional in lacking a highly ordered fibrous "cuticle" associated with its integumental epithelium (Storch and Welsch, 1970). The thinness of the membrane, together with the apparent absence of cuticular fibers (a viscous element), is probably crucial to the remarkable agreement between our almost assuredly too simplistic theory and the actual experimental results.

Thirdly, we do not propose that our mechanical "volumestat" model is the whole answer in isoosmotic volume-regulating worms—indeed, it cannot be in those animals (possibly a majority) where viscous integumental elements predominate. In addition, it would seem unlikely that the evolutionary process would yield only a single mechanism for volume-regulation in polychaetes, or possibly for even a single species.

Nevertheless, it is clear that in any worm having a permeable outer integument which is even partially elastic, a saline efflux as outlined above will result. It may prove profitable to take this particular aspect into consideration in future investigations into the problem of volume-regulation.

We are indebted to Dr. Rimmon C. Fay for his collecting efforts on our behalf, and to Mr. Edward Donnelly for his able assistance in maintaining the animals. This investigation was supported in part by New York State Research Foundation Grant-in-Aid 20-7117 to S. C. Brown; National Science Foundation Grant GA-27700 to J. B. Bdzil; and National Science Foundation Grant GP-19881 and American Chemical Society Petroleum Research Grant 3519C56 to H. L. Frisch.

SUMMARY

1. In *Chaetopterus* gradually adapted to lowered salinities, coelomic fluid osmolarity and sodium and chloride concentrations conform to ambient seawater at salinities from 1100 to 630 mOsm.

2. Experimental animals were unable to tolerate salinities below 630 mOsm for extended periods of time.

3. Worms transferred directly to hypoosmotic stress solutions down to 681.8 mOsm gained weight (volume) rapidly, reaching a maximum in approximately 1.5 hours. At this point, the coelomic fluid was isoosmotic with the external medium. After 1.5 hours, there followed a decrease in volume, which as shown in one experiment, culminated in a return to initial volume values.

4. Transfers back to full-strength seawater indicate that salt efflux as well as water influx occurred.

5. It is concluded that *Chaetopterus* is a volume-regulating osmoconformer over its viable range of salinities.

6. Current theories of the mechanism of isoosmotic volume-regulation are discussed and a mechanically linked "volumestat" model is proposed.

7. The behavior of the model system is mathematically analyzed utilizing the concepts of non-equilibrium thermodynamics.

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OXYGEN CONSUMPTION IN ANTERIOR *VERSUS* POSTERIOR EMBRYONIC SHIELD OF *FUNDULUS HETEROCLITUS*

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It has been demonstrated (Brummett, 1968, 1969) that the embryonic shield of *Fundulus* has the fate of its various organs and tissues determined early in gastrulation. When the anterior shield is excised and implanted into the pericardial chamber of an older embryo it differentiates brain and eyes; posterior shield, similarly handled, forms spinal cord, somitic muscle, gut, and other structures appropriate to trunk and tail. Histological studies of these tissues at the time of excision reveal no recognizable differences between the cells of the two shield regions. How, then, might one elucidate and perhaps characterize the differences which the transplantation experiments indicate to be present?

Promising approaches to the above question might include a comparison of the two regions in terms of their fine structure, their biochemistry, and their physiology. The experiments reported here represent an attempt to provide information on one aspect of the third of these approaches.

Differences in respiratory activity of various parts of an egg or developing embryo have long been accepted as indications of differences in extent and direction of differentiation. Barth and Sze (1951) and Sze (1953), for example, measured respiration in various parts of the anuran (*Rana pipiens*) gastrula and established respiratory gradients in that embryo which they related to both cellular interactions (induction) and differentiation.

The objective of the study presented here was to determine whether measurable differences in respiration exist between the anterior and the posterior embryonic shield of the gastrula of the teleost, *Fundulus heteroclitus*.

MATERIALS AND METHODS

Eggs of *Fundulus heteroclitus* were fertilized in the laboratory. To reduce the possibility of genetic variability in developmental rates, gametes were obtained from one male and one female for each set of experiments. Following fertilization, the eggs were kept at room temperature (approximately 24° C). When the eggs reached the desired stage of gastrulation [Oppenheimer (1937) stages 12, 14 and 16 which correspond to Armstrong and Child (1965) stages 15, 18, and 20], they were washed in 3% formalin-sea water to reduce the possibility of contamination, rinsed in filtered sea water, and dechorionated in sterile Tyrode solution (without bicarbonate) according to methods previously described (Brummett, 1968).

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Operating in sterile Tyrode solution, portions of the embryonic shield were carefully excised with finely sharpened watchmaker's forceps (Brummett, 1968, 1969). Taking care to avoid transferring yolk or periblast along with the tissues, the excised tissue was picked up with a micropipette and placed in a small amount of Tyrode solution in a spotting plate depression. As quickly as possible, each piece was then transferred from the spotting plate to Cartesian diver respirometers in a medium of sterile Tyrode solution. The total volume of the divers ranged from 10–13 μl. All metabolic rate determinations were made at 25° C for a period of approximately two to two and one-half hours.

Operations for weight determinations were performed as above in Tyrode solution. The excised shield parts were rinsed briefly in distilled water to remove most of the balanced salt solution, transferred to a preweighed piece of plastic wrap (Cutrite brand) or aluminum foil, dried overnight at 110° C, and weighed on a microtorsion balance having a sensitivity of ±2 μg.

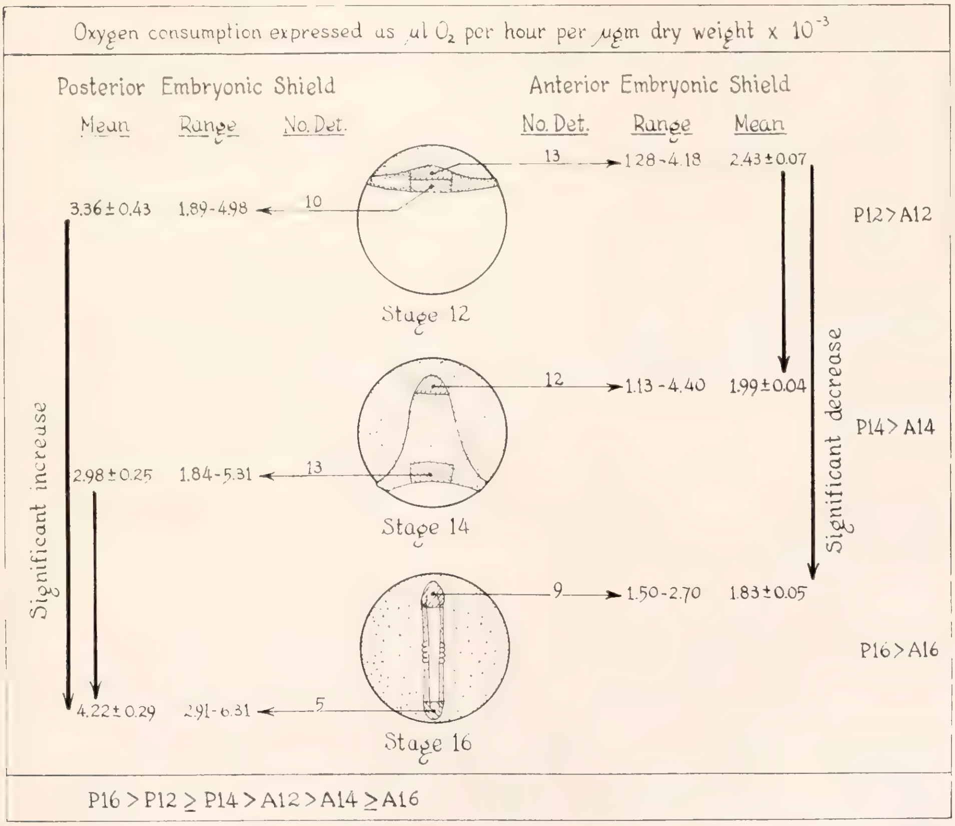
Oxygen consumption expressed as μl O₂ per hour per μgm dry weight x 10⁻³
Posterior Embryonic Shield
Mean
Range
No. Det.
Anterior Embryonic Shield
No. Det.
Range
Mean
13
1.28-4.18
2.43±0.07
3.36±0.43
1.89-4.98
10
P12>A12
Stage 12
12
1.13-4.40
1.99±0.04
Significant increase
Significant decrease
P14>A14
2.98±0.25
1.84-5.31
13
Stage 14
9
1.50-2.70
1.83±0.05
P16>A16
4.22±0.29
2.91-6.31
5
Stage 16
P16 > P12 ≥ P14 > A12 > A14 ≥ A16

FIGURE 1. Summary of O_2 consumption data for anterior *versus* posterior embryonic shield in three stages of development: early gastrula (stage 12), late gastrula (stage 14), and tail bud stage (16). The number of individual determinations made, the range of measurements obtained, the means ± standard errors for each pool, and significant increases and decreases are indicated.

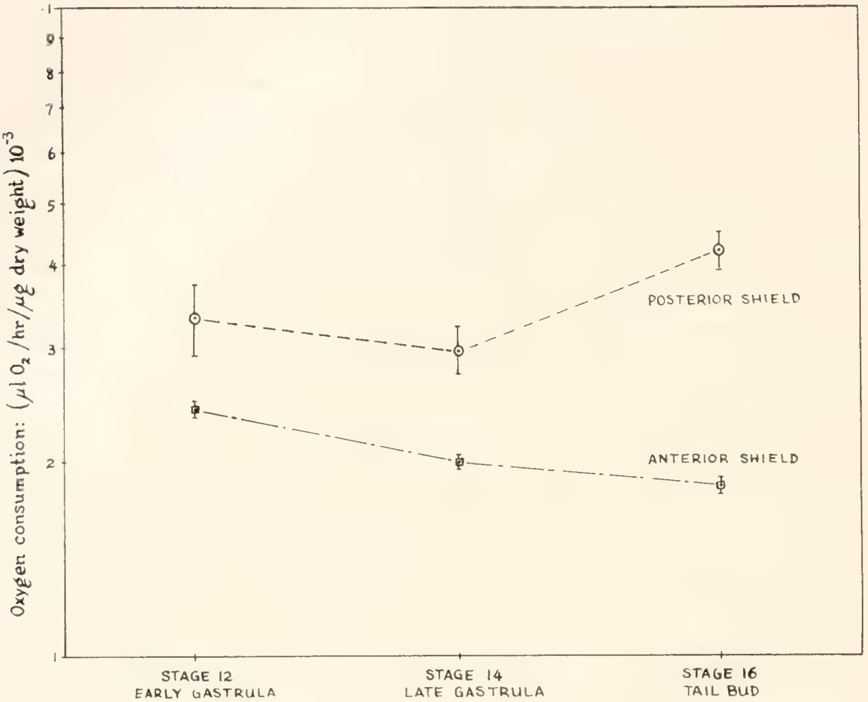

Figure 2. Graph comparing O_2 consumption data for anterior (⊡) *versus* posterior (⊙) embryonic shield of early gastrula (stage 12), late gastrula (stage 14), and tail bud stage (16) of *Fundulus heteroclitus* embryos. Standard errors are indicated in each case. Posterior shield measurements are significantly higher than anterior shield measurements at each stage of development and the difference increases as the embryo progresses from early gastrula to tail bud.

Results are expressed as μl O_2/hr per μg dry weight 10^{-3}. Significance of difference of means was determined by the method for small samples given in Simpson, Roe, and Lewontin (1960).

Results

Results are summarized in Figures 1 and 2. The metabolic rate of the posterior shield was significantly greater than that of the anterior shield in all three stages of development studied. Furthermore, these differences tended to increase as gastrulation proceeded: at early gastrula (stage 12) the metabolic rate of the anterior shield was 28% less than that of the posterior shield, at mid-gastrula (stage 14), 33% less, and immediately following blastopore closure (stage 16), 64% less. The level of oxygen uptake in the anterior shield tended to decrease with age; the rate decreasd 18% (significant at <1% level) between stages 12 and 14 and approximately 8% (not significant) between stages 14 and 16. In contrast, oxygen consumption in the posterior shield decreased 11% (not significant) between stages 12 and 14 but increased approximately 42% between stages 14 and 16 (significant at the <1% level).

DISCUSSION

Since injured cells will cytolyze, and cytolysis itself will cause changes in O_2 consumption, it is important to point out that explants of the early *Fundulus* embryo can be maintained in Tyrode solution for two days or longer without apparent damage to the cells (Brummett, Haynes, and Pillsbury, unpublished data). The duration of these O_2 consumption experiments was usually less, and never more, than three and one-half hours from the time of excision of the embryonic regions under consideration to termination of the experiment. It seems reasonable to assume then, that the health of the explanted tissues is not a problem in these experiments.

Anterior embryonic shield

The progressive decrease in oxygen consumption in anterior shield fragments as the shield progresses from stage 12 to stage 16 is not consonant with the notion that metabolic rate and hence energy requirement increases as differentiation progresses along a time axis. One is forced to consider other changes in the embryo which might explain the data. One possibility which suggests itself to the authors involves the idea of a progressive decrease in the *movement* of cells in the anterior shield accompanied by a decrease in metabolic rates during this developmental period. Earlier grafting experiments (Brummett, 1969) indicate that at stage 12 the anterior embryonic shield is composed of presumptive forebrain and retinal tissue. Such tissue, excised and implanted into the pericardial chamber of an older embryo, had differentiated brain in 100% and retina in 27% of the sectioned grafts. However, 37% of the *donors* of that tissue developed in an apparently normal fashion and only 13% were totally lacking forebrain and eyes. These results suggest that cells are in the process of migrating into the anterior shield during and perhaps subsequent to early gastrula (stage 12). Such an interpretation is in agreement with the data reported by Ballard and Dodes (1968) on the trout embryo where disengaged cells below the cellular envelope are described as moving into the anterior shield from the central region of the blastodisc during the early stages of epiboly: "As seen from below and confirmed in sagittal sections, the anterior and lateral edges of the gathering embryonic shield, even at six days, still grades off gently and smoothly into the thinned areas of the blastodisc. The convergence movements which bring together the prospective forebrain area of the shield become conspicuous at seven days, whereupon this part of the shield develops sharp boundaries. The outlying thinned area is by then two cells thick, consisting of the cellular envelope and one layer of inner cells" (Ballard and Dodes, 1968, page 77).

Extensive study of developing *Fundulus* embryos suggests that, except for the time factor (the smaller egg of *Fundulus* develops much more rapidly than the trout egg), the quoted description of anterior shield formation in the trout is consonant with the situation in the embryo of *F. heteroclitus*. It seems reasonable to suppose, then, that in early stages of gastrulation there would be considerable movement of cells comprising the early anterior embryonic shield and that, as the shield becomes more definitely formed, the movement would decrease and total oxygen consumption might decrease concomitantly. Significant decrease in cell movement would presumably obscure any increase in metabolic rate associated with progressive differentiation in the stages under consideration here.

Posterior embryonic shield

The increase in oxygen consumption in the posterior embryonic shield during gastrulation is perhaps more difficult to explain. It is almost certain that cells are actively moving into the posterior shield from the lateral shield at both stage 12 and stage 14 (Brummett 1954), and perhaps the amount of activity is essentially the same at these two stages. It seems unlikely, however, that such movement would be *greater* at stage 16 after blastopore closure and the formation of the definitive tail bud. Differentiation has no doubt proceeded further at stage 14 than at stage 12 and still further at stage 16, but in view of the anterior shield picture it would seem presumptuous to attribute the significant increase in oxygen consumption to what would appear to be minor differentiative changes. The question of differential growth comes to mind, but increased mitotic activity in the tail bud blastema must also be set aside as an acceptable explanation of the increase in oxygen consumption in this tissue if one accepts as pertinent Pasteels' (1943) results which revealed essentially no differences in mitotic activity when the blastoporal region was compared with the anterior shield region of the trout embryo. However, this possibility will be considered further below.

Comparison of anterior versus *posterior shield*

The phenomenon of early morphogenesis of brain, common in vertebrate embryos, might lead one to expect earlier and more extensive differentiation in the anterior region of the embryonic shield and hence, perhaps, a higher metabolic rate there than in the posterior shield. Such an expectation is not consistent with the results of these experiments, however, since the posterior shield exhibits a higher rate of oxygen consumption at all three of the stages measured, and the difference increases as gastrulation proceeds. The data suggests, then, that the anterior shield is not significantly ahead of the posterior shield along the differentiative pathway. This interpretation is reasonable in the light of the results of grafting experiments (Brummett, 1968, 1969) which reveal similar time scales for graft differentiation whether the tissue is derived from the anterior or posterior shield. Both donors and grafts of the earlier experiments also demonstrate that even at stage 12, the posterior shield is determined to form trunk and tail structures and the anterior shield is determined to form primarily forebrain, midbrain, and associated structures; presumptive hindbrain is intermediate in position and may be excised with either an anterior or a posterior stage 12 graft. In other words, determined cells appropriate to the entire axis are already present in the early embryonic shield. There is no evidence that presumptive head structures are present first (and hence are differentiatively older), and that presumptive trunk and tail structures enter the shield later as epiboly progresses and the shield elongates by virtue of the convergence of cells from the lateral shield and germ ring. With respect to the time axis of differentiation, then, anterior and posterior shield are, in all probability, differentiating simultaneously; *i.e.*, no one area exhibits a precocity of differentiation when compared with other areas of the developing embryo.

The preceding discussion is germane to that aspect of the results which shows that the rate of oxygen consumption in the anterior shield is *not higher* than in the posterior shield. It does not, however, respond to the opposite side of the question:

i.e., why is oxygen consumption in the posterior shield significantly *higher* than in the anterior shield?

It was suspected that differential growth rate might possibly be the source of the observed difference. With this idea in mind the data recorded in the earlier grafting experiments were reviewed, and the time at which obvious growth was first observed and recorded for individual living grafts was noted. When this information for the two series of experiments was compared, it became apparent that in the majority of those cases in which such information was recorded, the posterior grafts exhibited an increase in size *earlier* than did the anterior shield grafts. Indeed, during the first 24 hours following transplantation, growth was noted in 71% (50 of 71 cases) of the posterior grafts as compared with only 8% (3 of 37 cases) of the anterior shield grafts (see Table I). Since the earlier experiments were not designed with this particular question in mind, the records are not as complete as one might wish, but the data agree with the notion that differential growth rate may contribute to higher oxygen consumption in the posterior shield.

TABLE I

Tabulation of growth data (the time when increase in size of graft was first noted) recorded in earlier experiments for anterior and posterior embryonic shield grafts. In general, posterior grafts exhibited increase in size earlier in the post-operative period than did anterior shield grafts

	Anterior embryonic shield grafts					Hrs post-operation	Posterior embryonic shield grafts					
	Totals # (%)	St. 12 # (%)	St. 13 # (%)	St. 14 # (%)	St. 15 # (%)		St. 12 # (%)	St. 13 # (%)	St. 14 # (%)	St. 15 # (%)	Totals # (%)	
8%	0 —	0 —	0 —	0 —	0 —	6–12	1 (11)	7 (37)	9 (28)	4 (36)	21 (30)	71%
	3 (8)	0 —	3 (19)	0 —	0 —	18–24	5 (55)	4 (21)	14 (44)	6 (55)	29 (41)	
	2 (5)	1 (8)	1 (6)	0 —	0 —	30–36	2 (22)	2 (11)	6 (19)	0 —	10 (14)	
	10 (27)	2 (15)	4 (25)	1 (20)	3 (100)	42–48	1 (11)	5 (26)	1 (3)	1 (9)	8 (11)	
	6 (16)	2 (15)	3 (19)	1 (20)	0 —	54–60	0 —	0 —	2 (6)	0 —	2 (3)	
	16 (43)	8 (62)	5 (31)	3 (60)	0 —	66–72	0 —	1 (5)	0	0 —	1 (1)	
	37	13	16	5	3		9	19	32	11	71	

Mitotic counts should be made to substantiate this interpretation. As was mentioned earlier, however, mitotic counts on the trout embryo by Pasteels (1943) produced negative results.

It is of interest to note that in her studies of metabolic gradients in teleost embryos, Hyman (1921) found that in *Fundulus heteroclitus* the pattern of differential susceptibility to toxins always began at the posterior end of the embryonic axis and proceeded anteriorly. A second susceptibility gradient began subsequently at the anterior end of the axis. Hyman does not provide a great deal of data but what is presented is in agreement with that presented in this paper. Hyman interprets her data to mean that the region of high activity at the posterior end of the embryonic axis reflects a growing point which is responsible for the laying down of the greater part of the *Fundulus* embryo.

Comparison of these results with similar experiments on the amphibian gastrula

Using essentially the same methods described in this paper, Sze (1953) measured oxygen consumption in various portions of the early gastrula of the frog, *Rana* *pipiens.* The results of those experiments indicate that oxygen consumption in the frog embryo is less than that in the *Fundulus* embryonic shield by a factor of ten. This is not surprising since the cells of the early amphibian embryo contain a considerable amount of stored yolk which is, presumably, metabolically inert. The cells of the teleost embryo, on the other hand, contain little or no stored yolk but appear to absorb it as needed through the periblast membrane which separates the developing embryo from direct contact with the yolk.

In this study Sze (1953) found that the presumptive neural regions of the early frog gastrula exhibited a higher rate of oxygen consumption than did the presumptive chordamesoderm. Deletion-grafting experiments (Brummett, 1968) demonstrate that the posterior shield tissue in *Fundulus* is not limited to chordamesoderm but consists, rather, of cells which are already determined to form *all* tissues appropriate to posterior trunk and tail. The regions of the frog gastrula measured by Sze (1953), then, are not directly comparable to the teleost tissues used in the experiments reported here.

In another series of experiments in which parts of the frog gastrula in various combinations were subjected to respiration measurements, Barth and Sze (1951) attributed increased oxygen consumption to inductive interactions between "organizer" and presumptive neural plate or presumptive epidermis. Although the teleost posterior embryonic shield has for many years been homologized with the dorsal lip region of the early amphibian embryo, convincing evidence that it is an "organizer" region is lacking. The posterior embryonic shield of the *Fundulus* embryo, however, does contain a wider variety of presumptive tissues than does the anterior shield as revealed by the differentiation of grafts from these two regions (Brummett, 1968, 1969). Nothing is known concerning possible inductive interactions among these various components of the posterior shield in the teleost embryo, but of possible significance is the fact that presumptive notochord appears to be present in the posterior embryonic shield from stage 12 on (Brummett, 1968). In contrast, notochord *never* differentiated in anterior shield grafts of stages 13, 14, and 15, and was found to be present only in those stage 12 grafts which had included more than half of the very early shield (Brummett, 1969). Perhaps, then, the results of the experiments reported here can best be explained in terms of energy-consuming cell interactions (including induction?) which may be significantly greater in the posterior than in the anterior shield at all stages of gastrulation, which may increase in the posterior shield as the embryo goes from stage 12 to stage 16, and which may decrease in the anterior shield during the same period.

It is felt that the experimental data presented in this paper is convincing evidence that measurable physiological differences exist in the posterior *versus* the anterior embryonic shield at a stage when morphological differences in the cells, at the level of magnification possible with light microscopy, is lacking. Electron microscope studies on these tissues are in progress in the hope that they might provide further elucidation of the question of differences. Cell movements, cell proliferation, cell interactions, and cell differentiation are undoubtedly occurring simultaneously in the tissues which were excised and measured in these experiments. These various aspects of cell activity no doubt vary quantitatively in different regions of the embryo and in the same region at different stages of development. They may also vary considerably in their energy requirements. It is difficult, then, to delineate accurately the causal factors responsible for the differences in oxygen consumption demonstrated by these experiments. We have attempted in the discussion above, however, to consider the possible contribution of each of these important aspects of cell activity to the differences in O_2 uptake obtained in these experiments, and we have tried to interpret the results in the light of what is known about the teleost embryo at this stage of development.

SUMMARY

1. Experiments were designed to determine whether measurable differences in respiration exist between the anterior and posterior embryonic shield of the gastrula of *Fundulus heteroclitus.*

2. Anterior and posterior embryonic shields were carefully excised from embryos of early gastrula, late gastrula, and post-blastopore-closure stages. Using Cartesian diver microrespirometers (10 to 13 μl), oxygen consumption of each individual explant was measured at 25° C over a period of two to two and one-half hours.

3. Oxygen consumption data, expressed as μl O_2 per hour per μg dry weight 10^{-3}, for the two embryonic shield regions at the three stages of development are compared.

4. Posterior shield was found to exhibit a significantly higher rate of O_2 uptake than anterior embryonic shield at all three stages of development.

5. Oxygen uptake appears to increase in the posterior shield as development proceeds from early gastrula to closure of the blastopore; anterior shield exhibits a concomitant decrease.

6. The results are discussed in light of what is known about cell movements, cell proliferation, cell interactions, and cell differentiation in the teleost embryo and are compared with similar experiments on amphibian embryos.

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TEMPERATURE, WATER, AND RESPIRATORY REGIMES OF AN AMPHIBIOUS SNAIL, *POMACEA URCEUS* (MÜLLER), FROM THE VENEZUELAN SAVANNAH

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It has long been known (Troschel, 1845; Pelseneer, 1895; Prashad, 1925, 1932) that ampullariid or "pilid" snails possess "amphibious" respiratory structures, one part of the mantle cavity containing a ctenidium (the characteristic molluscan gill) and another part being modified as a gas-filled lung cavity. This double adaptation for aquatic and aerial respiration in *Pomacea urceus* is probably most important during the rainy season (Burky and Burky, in preparation). Respiration is amphibious in both its structural (Andrews, 1965) and its behavioral (Burky and Burky, in preparation) aspects. In addition, the annual sequence of rainy and dry seasons of the Venezuelan plains dictates an amphibious way of life for *Pomacea urceus* (Burky, in preparation).

Terrestrial snails face particular problems of temperature regulation and water balance since their moist bodies are exposed during activity (Howes and Wells, 1934a, 1934b; Hogben and Kirk, 1944; Dainton, 1954a, 1954b; Russell Hunter, 1964; Machin, 1964, 1965, 1966; Cloudsley-Thompson, 1968, 1970; Vernberg and Vernberg, 1970). The relationship of body temperature to environmental temperature has been reported for intertidal (Lewis, 1963; Fraenkel, 1968; Grainger, 1968; Davies, 1970; Newell, Pye, and Ahsanullah, 1971; Vermeij, 1971; and others) and for other terrestrial gastropods (Hogben and Kirk, 1944; Dainton, 1954a; and others). In deserts and in tropical areas with periodic dry seasons there are additional problems of high temperatures and limited water. Under arid conditions the body temperatures of the pulmonates *Helicella virgata* (Pomeroy, 1968) and *Trochoidea seetzeni* (Yom-Tov, 1971a) are a function of their position in the temperature gradient between air and ground as they aestivate attached to vegetation above the ground. When the pulmonate *Sphincterochila boissieri* is dormant on the ground surface, body temperature is primarily a function of slow conduction from the substrate and the transfer of body heat to the air (Yom-Tov, 1971a; Schmidt-Nielsen, Taylor, Shkolnik, 1971). For these pulmonates, the rate of water loss is too low to be of value for evaporative cooling. In desert snails the high reflectivity of light-colored shells is important in reducing the absorption of solar radiation (Yom-Tov, 1971a; Schmidt-Nielsen *et al.*, 1971). Some snails wait out severe climatic periods in the ground (Pain, 1950; Meenakshi, 1964; Coles, 1968; Pomeroy, 1968; Yom-Tov, 1971a, 1971b; Schmidt-Nielsen *et al.*, 1971; and others). In aestivating ampullariids, *Pomacea lineata* can tolerate a loss of over 50% of its tissue weight (Little, 1968) while *Pila virens* dies when 50% of its tissue water has been lost (Meenakshi, 1964). Also, metabolism during aestivation in snails belonging to the family Ampullariidae has received attention. The Indian ampullariid, *Pila virens,* has been shown to be anaerobic (Meenakshi, 1956, 1957, 1964) and the African ampullariid, *Pila ovata,* has been shown to be aerobic (Visser, 1965; Coles, 1968, 1969) during aestivation. Such differences raise questions about metabolism as well as water economy and temperature regulation in a neotropical ampullariid like *Pomacea urceus.*

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Since life history, growth, and biomass production (Burky, in preparation), and buoyancy changes as related to respiratory behavior (Burky and Burky, in preparation) were being studied for *Pomacea urceus,* it was decided to investigate the temperature and water regimes during aestivation and to measure oxygen consumption in active and aestivating snails. The adaptive significance of the data on temperature, water loss, and oxygen consumption is discussed in relation to the annual dry season and to the other existing information on *Pomacea urceus* (Burky, in preparation; Burky and Burky, in preparation).

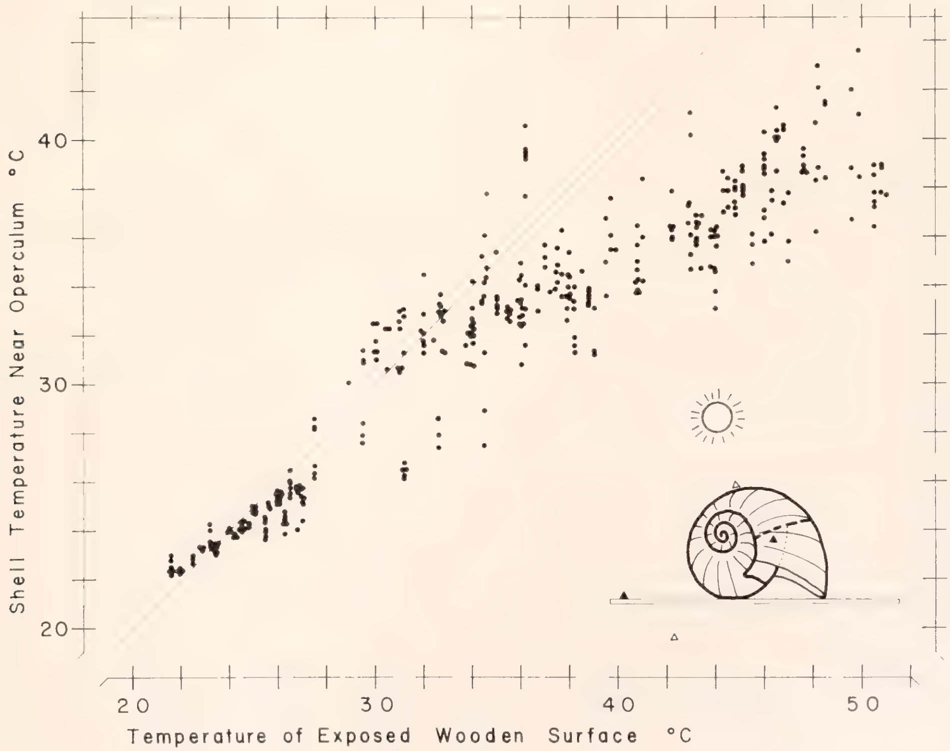

FIGURE 1. Shell temperature near the operculum of *Pomacea urceus* is plotted against the temperature of the exposed wooden surface; snails are experimentally exposed to direct sun during aestivation. The isothermal line is given for reference. The stylized diagram gives the position of an experimental snail in relation to sun and wooden surface. The closed triangles represent the positions of the thermistor probes for the plotted data. The open triangles give the positions of thermistor probes recording the temperatures of shaded air and upper exposed shell surface. For further details, see text.

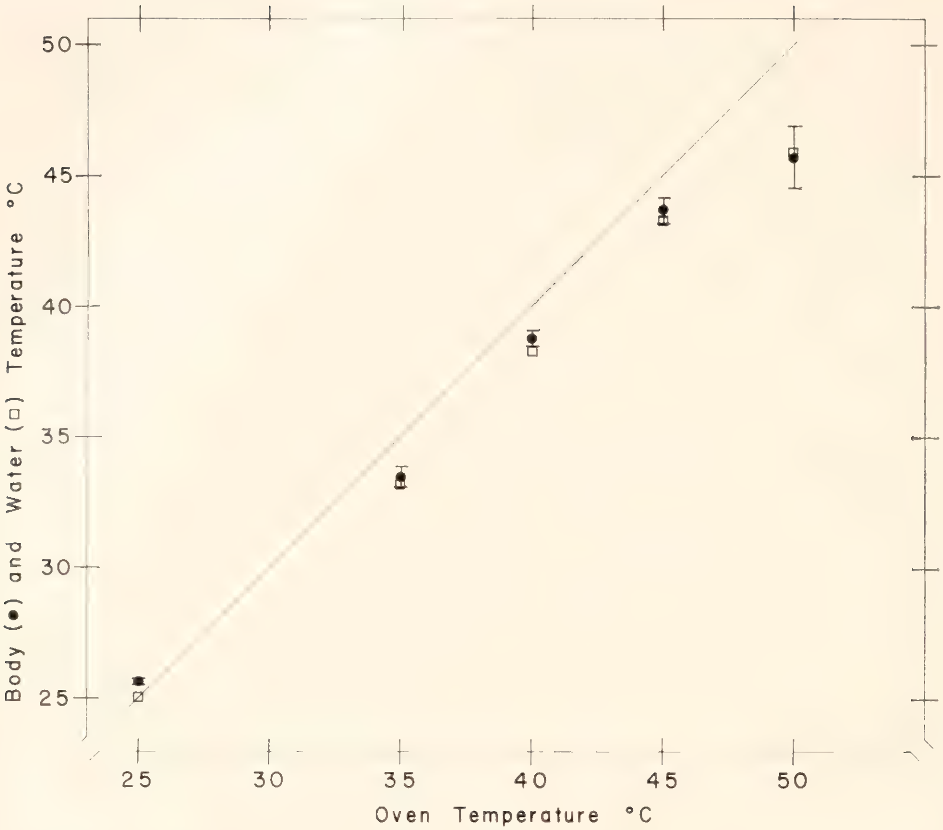

FIGURE 2. Body temperature of *Pomacea urceus* (closed circles) with 95% confidence intervals and the temperature of water in an open beaker (open squares) are plotted against oven temperature. The isothermal line is given for reference. For further details, see text.

MATERIALS AND METHODS

Pomacea urceus was collected in the plains (Los Llanos) region of Venezuela. Field studies were carried out in the local area known as El Estero de Camaguán near the village of Camaguán in Guarico State. Laboratory experiments on temperature and oxygen consumption were carried out on aestivating and active snails collected during the dry and rainy seasons, respectively.

Snails were experimentally exposed to the tropical sun to record their response to the absorption of solar radiation. Aestivating adult snails were placed (aperture opening downward) on a gray wooden surface on a roof terrace of the laboratory in Caracas. Experiments were set up in the morning before the sun was high enough to directly illuminate the experimental area (initially the snails were in the shade). In this way the snails were gradually exposed to the direct tropical sun. During the day the major influences on solar radiation were the passage of clouds and the angle of the rays as the position of the sun changed. Temperatures of shaded air, exposed wooden surface about 25 cm from the snail, upper exposed shell surface, and lower shaded shell surface (columella next to the operculum) were recorded at 20 minute intervals with YSI thermistor probes. The position of these probes is indicated in Figure 1. The shell temperature next to the operculum was chosen as an index of body core temperature because this area was shaded; it was *not* in contact with the wooden surface; it was adjacent to a large volume of tissue; and the probe could be taped to the shell without disturbing the snail or obstructing the movement of the operculum. During aestivation the operculum of a large snail can be withdrawn 3 to 6 cm from the aperture edge of the columella area. At the end of some experiments a small hole was made in the shell (using a ballpeen hammer) and core temperature was recorded (less than 30 seconds needed for this manipulation) with a hypodermic thermistor probe. These core temperatures can then be compared to temperatures taken next to the operculum. The core temperatures of 12 snails average 1.2° C higher than the columella shell temperature (core index) of Figure 1.

Snails were maintained at controlled temperatures so their body temperature could be compared with the temperature of water having a free surface. Groups of five to eight adults were maintained for four hours or longer at oven temperatures of 25, 35, 40, 45, and 50° C. Shell temperature and temperature of water in an open beaker (100 ml—roughly equivalent to snail tissue volume) were recorded at 20 minute intervals until shell temperature was constant for three successive readings. Body core temperature was taken at the end of each experiment; mean values with 95% confidence limits are given in Figure 2.

Field temperatures were taken before the end of the dry season and recorded with YSI thermistor probes. The body core temperatures were taken only after all other values had been recorded. The snails were removed from their positions of aestivation, a hole made, and the thermistor probe inserted (less than one minute needed for this manipulation). The soil moisture content was measured with a soil moisture meter (O.S.K. 450 Riken Moisture Meter, Model R-1-1 Moisture Indicator), a calibrated conductivity determination. These field values are summarized in Table I.

Weight loss during aestivation was measured as an index of tolerance to water loss. For weighing experiments snails were initially maintained in large outside tanks and fed lettuce. Active adults (males and females) were removed from these tanks, drained, weighed, and placed in open cardboard boxes to initiate aestivation. Snails were maintained in the laboratory at room temperature, generally between 20 and 25° C, and weighed at varying intervals. The snails were usually weighed over a period of five days, so a mean date was determined for each weighing. A long term and a short term experiment were started in January 1969 and in February 1970, respectively. Each snail's weight is expressed as a percentage of its weight on the first day of aestivation. The mean percentage and 95% confidence limits have been computed for the snails of each weighing.

The oxygen consumption of aestivating and active snails was measured at 30° C using two plexiglass chambers (1.6 liter) and Warburg manometers (KOH was used for CO_2 absorption). One to four aestivating snails could be placed in a chamber while only one active snail could be measured in an experiment. Thirty minutes to an hour was allowed for equilibration and one chamber was used as a thermo-barometer. Readings were taken at about 30 to 60 minute intervals. After each experiment the shell length, whole animal live weight, body wet weight (no shell), and body dry weight (100° C in an oven until constant weight) were determined (Table II).

RESULTS

The relationship of columella shell temperature near the operculum (index of core temperature) to exposed wooden surface temperature (25 cm from shell) is shown for 25 individual adult snails (males and females, shell lengths 94 to 129 mm) in Figure 1. The temperatures near the operculum (core index) for exposed wooden surface temperatures above 24–25° C (Fig. 1) were recorded after the snails were no longer in the shade. Those points above the isothermal line were recorded after clouds had obstructed solar radiation and indicates that the wooden surface cools faster than the shell near the operculum (core index). Throughout these experiments the temperature of the upper exposed shell surface (data not given, see diagram of Figure 1) were higher than the temperatures of the exposed wooden surface, due to heat absorption by the dark brown to black coloring of the snail shells. Shaded air temperature (data not given, see diagram of Figure 1) were always 8-10° C lower than those of the shell near the operculum (core index) at higher temperatures (above 35° C). This indicates some heat flow from the snail to the air. During these experiments on aestivating adults, opercular movements (exposure of mantle tissue to air) were commonly observed, particularly at higher temperatures when evaporative cooling can be assumed to be important. All snails survived these experiments. The data of Figure 1 indicates regulation of body temperature, generally below 41° C when animals are experimentally exposed to direct solar radiation.

Figure 2 gives the results of experiments at controlled air temperatures in an oven. The higher body temperatures at 25° C in the oven might be a factor of metabolic heat production. The body core temperatures at oven temperatures of 35, 40, and 45° C were about 0.5° C higher than the water temperatures of open beakers. Snails at 40° C remained in good condition (opercular movements observed) while the bodies of those at 45° C were extended to the edge of the aperture opening with foot tissue visable. The snails at 45° C didn't retract rapidly when touched. The bodies of the snails at 50° C were extended beyond the edge of the shell aperture; they were either moribund or dead after a few hours. This condition is reflected in the large variation in the body temperature of snails at 50° C (Fig. 2) with a mean core temperature which is essentially the same as the temperature of water in an open beaker. These experiments at controlled temperatures indicate an upper lethal temperature between 40 and 45° C and show that evaporative water loss is responsible for lowering body temperature (Fig. 2).

Four groups of 25 aestivating spat (mean shell length about 10 mm and mean weight about 0.3 g) were exposed to the mid-day tropical sun in April 1970. Within 30 minutes all had fluid bubbling from the edges of their opercula. At the end of two hours they were submerged in water and all were dead. Another group (150 spat) was not exposed to the sun and all were active within one hour of submersion in water, some within three minutes. Significantly, adults were exposed to the sun for many days without mortality. Exposure in the field is discussed below.

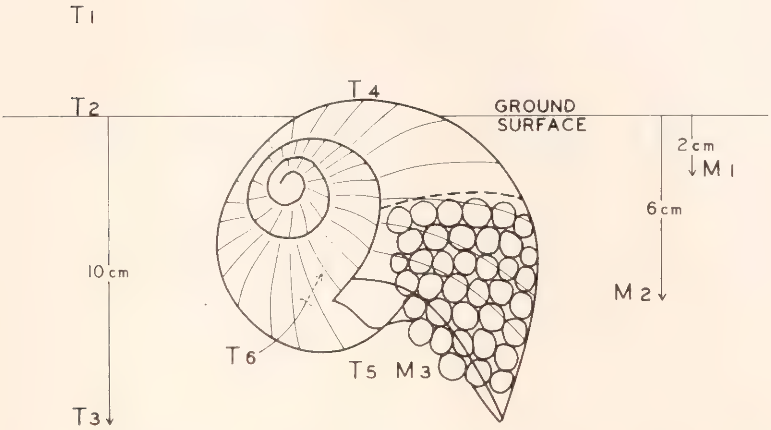

FIGURE 3. The position of a female *Pomacea urceus* (stylized shell) with eggs during aestivation in the field. T_1 to T_6 (°C) are reference points for temperatures given in Table I and correspond to shaded air, ground surface, soil (10 cm depth) about 25 cm from snail, exposed shell surface, soil beneath snail, and body core temperature respectively. M_1, M_2, and M_3 are reference points for the soil moisture content values of Table I. The depths, shell, and eggs are drawn to scale.

TABLE I

Temperature and moisture regimes for Pomacea urceus *during aestivation in the savannah. Temperature (°C—T_1 to T_6) and soil moisture content (weight ratio—M_1 to M_3) values correspond to the reference points of Figure 3*

	Morning (8:15–9:30) May 2, 1970			Mid-day (12:30–14:45) May 1, 1970		
	Range	Mean	±95% confidence limits	Range	Mean	±95% confidence limits
T_1	31.5–34.0	32.71	± 1.128	35.0–40.0	38.17	± 1.231
T_2	32.5–38.5	35.57	± 2.511	42.5–54.5	49.61	± 3.012
T_3	27.3–30.2	28.66	± 1.343	29.6–33.7	32.02	± 0.889
T_4	32.0–36.5	34.09	± 1.880	42.0–56.5	48.69	± 3.098
T_5	27.2–28.6	27.97	± 0.506	31.6–37.5	34.01	± 1.447
T_6	27.4–30.7	28.91	± 1.198	34.6–39.3	37.51	± 1.138
M_1	40–50	44.6	± 4.23	25–78	45.9	±15.17
M_2	50–80	70.6	±13.02	55–82	70.3	± 6.37
M_3	69–74	72.0	± 2.14	49–84	65.9	± 7.55

Values for temperatures and soil moisture near *Pomacea urceus* aestivating in the savannah are given in Table I for the reference points of Figure 3. Measurements were taken on seven snails (mean shell length, 116.7 mm ± 9.01 S.D.) during the morning and on nine snails (mean shell length, 105.3 mm ± 10.77 S.D.) during the afternoon. Adult snails normally aestivate with a small patch of shell surface exposed (Fig. 3), and the hard-baked ground has small cracks which apparently form minute air passages to the brood chamber beneath the shell aperture. Figure 3 shows the position of an egg clutch at the beginning of the dry season. At the time of these field measurements (end of dry season), only spat were found beneath females. Soil moisture content increases with depth. However, in the afternoon the soil moisture content immediately below the snails (about 10 cm depth) is somewhat less than at 6 cm. This lower moisture content would suggest evaporative water loss from the soil adjacent to the snails; however, these differences are not significant (Table I). This does not mean that evaporative cooling by the soil is not important. It was not possible to take soil moisture readings at 10 cm depth for comparison with the level immediately below the snails. Also, a certain amount of variation is implicit in these field studies since snails were found aestivating at varying distances from areas of standing water, and these measurements were taken over periods of one hour 15 minutes and four hours 15 minutes during the morning and afternoon, respectively. At all times the temperature of exposed shell (T_4) was higher than body core temperature (T_6) due to the absorption of solar heat. The difference between T_4 and T_6 indicates transfer of heat from the shell surface to the body tissues. In the morning the soil temperature beneath snails (T_5) is generally less than the soil temperature at 10 cm depth (T_3), thus indicating evaporative water loss (cooling) of the soil adjacent to the snails. In the afternoon the relationship is reversed with T_5 greater than T_3 indicating transfer of body heat to the surrounding soil at a time of greatest solar absorption. The high shell temperature is a result of the dark brown to black coloration (significance will be discussed). Also, the temperature of exposed shell surface (T_4) is greater than the air temperature (T_1) indicating heat transfer from the exposed shell surface to the air.

TABLE II

Oxygen consumption for active and aestivating snails at 30°C

	Active	Aestivating
Shell length (mm) Mean ± S.D.	110.2 ± 10.64	104.7 ± 12.59
Live (body & shell) weight (g) Mean ± S.D.	294.5 ± 104.18	169.6 ± 64.34
Body wet weight (g) Mean ± S.D.	101.0 ± 36.37	58.6 ± 23.58
Body dry weight (g) Mean ± S.D.	20.7 ± 12.81	12.7 ± 4.42
ml O_2/hr/mean snail ±95% confidence limits	5.07 ± 1.458	1.05 ± 0.439
μl O_2/hr/g wet ±95% confidence limits	54.57 ± 15.289	20.82 ± 12.452
μl O_2/hr/g dry ±95% confidence limits	310.72 ± 73.551	91.81 ± 49.902

The weight loss during aestivation is given in Figure 4. A long term experiment on 126 snails (mean shell length, 91.5 mm; live weight range, 32.6–288.2 g at start) was terminated 526 days later with 83 living snails. A short term experiment on 41 snails (mean shell length, 100.3 mm; live weight range, 81.5–385.6 g

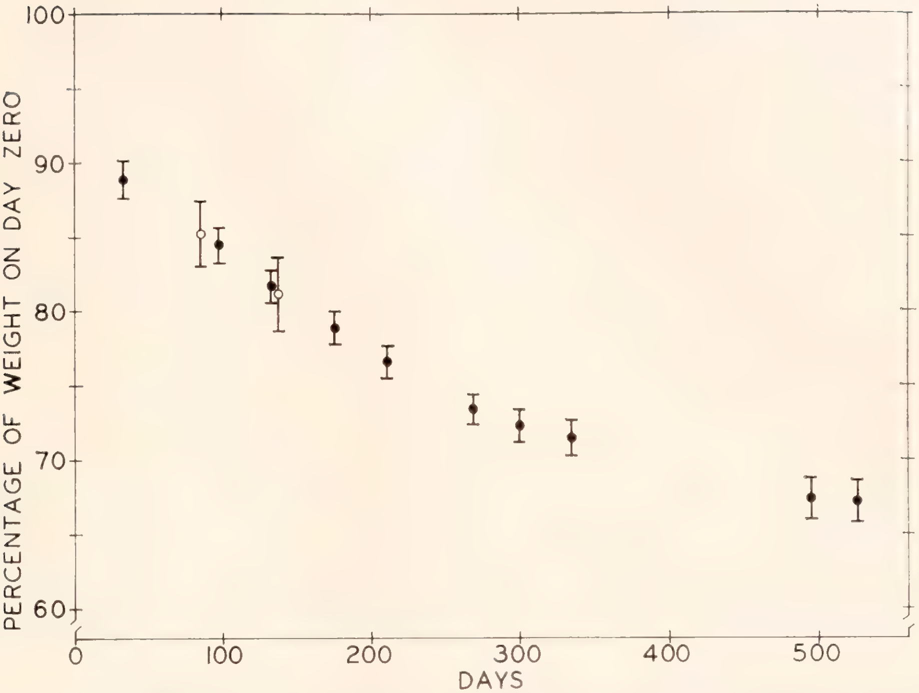

FIGURE 4. Weight loss during aestivation of *Pomacea urceus* in the laboratory is plotted against time. The mean weights of a long term experiment (closed circles) and a short term experiment (open circles) are given with 95% confidence limits. For further details, see text.

at start) was terminated 137 days later with 32 living snails. The loss of snails during the course of these experiments was due to mortality and the utilization of some specimens for other experiments (only one died in the short term experiment). The duration of the short term experiment approximates a normal period of aestivation (about 4.5 months) with 19% weight loss (about 35% of tissue weight). The long term survival (526 days) under these conditions with 34% weight loss (about 62% of tissue weight) is remarkable. This represents an aestivation period of about four times the normal. The initial weight loss is most rapid and represents the laying of eggs by some females and probably the loss of water retained in the mantle cavities of both males and females. On occasion, one of us (A. J. B.) recorded relative humidities as low as 45% and as high as 80% at the laboratory during the dry and rainy seasons, respectively. The weight loss during aestivation would give gross values of between 118 and 233 mg H_2O per day for a snail with a tissue weight of 100 g for the long and short term experiments, respectively.

The oxygen consumption values for 15 aestivating and 18 active *P. urceus* are summarized in Table II. Rates were measured at 30° C since this is within the normal range for body temperatures during aestivation (Table I) and during activity in the rainy season when the diurnal temperature ranges can be 28.6–32.8° C (Burky and Burky, in preparation). The total oxygen consumption for active adults is about five times greater than for aestivating snails. Also, the values in Table II draw attention to the marked differences in body weights for active and aestivating snails of similar size.

Five aestivating adult snails were placed in each of three sealed desiccators with dial hygrometers: (1) air and $CaCl_2$; (2) KOH, $CaCl_2$, pyrogallol, and replacement of air with N_2; (3) same as No. 2 but without KOH. In five days the chamber humidities had risen to 32, 44, and 41%, respectively with activity of two and three snails in chambers (2) and (3), respectively. On day 26 all snails in chambers (2) and (3) were dead. All snails in chamber (1) were living on day 77. Based on the oxygen consumption rates of Table II, chamber (1) contained more than the two liters of air necessary to supply oxygen for these snails. The mortality in the other two chambers indicate that *Pomacea urceus* can not survive for prolonged periods under anaerobic conditions.

Discussion

As pointed out in the introduction, respiration in ampullariid snails is amphibious. Although many ampullariids are completely aquatic, some are terrestrial for oviposition and/or for aestivation during periodic dry seasons (Prashad, 1925, 1932; Ranjah, 1942; Meenakshi, 1956, 1957, 1964; Coles, 1968; Little, 1968; Burky, in preparation; and others). It follows that temperature regulation for these snails is also amphibious. During the wet season, ampullariids are directly subject to the temperature fluctuations of the water. Prashad (1925, 1932) reports that *Pila globosa* commonly makes long excursions on land and will remain out of water for a few hours to lay eggs, but there is no information on temperature. Apart from this, information indicates that *Pila globosa* (Prashad, 1925, 1932; Ranjah, 1942; Saxena, 1955), *Pila virens* (Meenakshi, 1956, 1957, 1964), *Pila ovata* (Coles, 1968, 1969), *Pomacea lineata* (Little, 1968), *Pomacea depressa* (Little, 1968), and *Pomacea urceus* (Pain, 1950; Burky, in preparation, and this report) are in aestivation for most of their terrestrial phase.

This study shows that *Pomacea urceus* (adults) can regulate body temperature under experimental conditions (Figs. 1 and 2) by exposing foot and mantle through opercular movements. In pulmonate slugs there are additional problems of water loss since there is no shell for protection (Hogben and Kirk, 1944; Dainton, 1954a, 1954b). At controlled temperatures it is indicated that water evaporation from *P. urceus* may be similar to loss from a free water surface. Significantly, evaporative water loss for active *Helix aspersa* is similar to that of a free water surface (Machin, 1964, 1965), but water loss from the mantle of inactive *H. aspersa* can be regulated (Machin, 1965, 1966). Water loss for pulmonate snails under arid conditions is inadequate for temperature regulation (Pomeroy, 1968; Yom-Tov, 1971a; Schmidt-Nielsen *et al.*, 1971). For *Helicella virgata* (Pomeroy, 1968) and for *Trochoidea seetzeni* (Yom-Tov, 1971a) the climbing on vegetation is important for regulation of body temperature. This is a function of their position in the temperature gradient between soil and air. *Sphincterochilla boissieri* survives exposure to the sun on the ground surface of the desert by the high reflectivity of its light colored shell (Yom-Tov, 1971a) and by slow conduction of heat from the substrate (Schmidt-Nielsen *et al.*, 1971). Under field conditions the dark shell of *Pomacea urceus* absorbs solar radiation and heat is transferred to the air and to the body tissues. Heat then flows from the snail to its burrow walls by conduction and subsequently some heat is dissipated by evaporative water loss from the earth next to the shell (Fig. 3). The water loss during aestivation under experimental conditions (118–233 mg H_2O/day/100 g tissue) would be inadequate to be an important factor for sustained temperature regulation in the field. However, the ability to regulate temperature by evaporative cooling could act as a supplement to the cooling afforded by the ground. *Pomacea urceus* can survive 14 months with a loss of 62% of its tissue weight (35% during a normal dry season). *Pomacea lineata* can survive for over 13 months with a loss of greater than 50% of its tissue weight (Little, 1968). Normally, *Pila virens* loses 5% of its tissue water over a six month period, but under experimental conditions (operculum removed) snails die when 50% of their tissue water has been lost (Meenakshi, 1964). Aestivation in *Pila virens* is obviously different since it is found deep in the ground with the operculum and dried mucus sealing the shell opening (Meenakshi, 1964). Meenakshi (1964) also showed that *Pila virens* does not survive at or above 40° C, nor at or below 20° C. The present data on *Pomacea urceus* suggests an upper lethal temperature between 40 and 45° C with temperature regulation generally below 41° C (Table I; Figs. 1 and 3). A lower lethal temperature was not determined but it would not be surprising if it is similar to that found for *Pila virens* since the data of Burky and Burky (in preparation) and Table I indicate that field temperatures for *Pomacea urceus* are normally above 24–25° C.

Excretion must also be considered in water balance. It is known that *Pila ovata* (Visser, 1965), *Pila globosa* (Saxena, 1955; Raghupathiramireddy and Swami, 1963), *Pomacea lineata* (Little, 1968), and *Pomacea urceus* (Pacheco and Pereyra, unpublished data) all excrete uric acid during aestivation. For aestivating *Pomacea urceus* 4.2, 4.4, and 8.1 mg uric acid per gram of fresh tissue are found for hepatopancreas, foot, and kidney, respectively (Pacheco and Pereyra, unpublished data). The water saving advantage of uricotillism during aestivation is apparent.

The exposure of a small patch of the brown to black shell is a seemingly non-adaptive condition. It is known that many snails of deserts have light-colored shells (Morton, 1958; Russell Hunter, 1964; Pomeroy, 1968; Yom-Tov, 1971a; Schmidt-Nielsen *et al.*, 1971) and that this coloring acts to reflect solar energy (Yom-Tov, 1971a). Exposure of the shell by *Pomacea urceus* appears to promote the formation of cracks about the animal as the ground surface dries. These cracks undoubtedly aid in evaporative water loss from the area of the shell and for the diffusion of air (metabolism is discussed below). The dark sculptured shell has a cryptic function since it blends with the ground surface, *i.e.,* visual protection from predation by birds.

Oviposition in *Pomacea urceus* is at the beginning of the dry season after the female has burrowed into the surface mud. Clutches are laid beneath the shell aperture where the spat hatch and aestivate until the rains start about four months later. Camouflage and temperature regulation is apparently important for the protection of spat since they are unable to survive exposure to the tropical sun under experimental conditions (this report) or in the field during the dry season (Burky, in preparation). It is also known that adults reach a shell length of at least 85 mm before going into aestivation at the beginning of the dry season (Burky, in preparation). This suggests that there may be a minimum body volume for maintenance of body temperature and for water balance during the dry season.

Although aestivation in *Pila virens* is anaerobic (Meenakshi, 1956, 1957, 1964), it is aerobic in *Pila ovata* (Visser, 1965; Coles, 1968, 1969) and in *Pomacea urceus*. Further, *Pila virens* accumulates lactic acid during aestivation (Meenakshi, 1956, 1957) while *Pila ovata* (Coles, 1968) and *Pomacea urceus* (0.219 and 0.183 mg lactic acid per gram of fresh tissue for foot and hepatopancreas, respectively; Pacheco and Pereyra, unpublished data) do not accumulate lactic acid during aestivation. The special problems of animals under anaerobic conditions have been discussed (von Brand, 1946; Dales, 1956; Beadle, 1961; and others). Both *Pomacea urceus* and *Pila ovata* (Coles, 1968) have a similar rate of oxygen consumption with the rate during aestivation being one fifth and one sixth that for active snails respectively. In *Pila ovata,* aestivating snails elevate oxygen consumption when disturbed (Coles, 1968). This type of disturbance reaction is undoubtedly true for *Pomacea urceus* since aestivating adults have been observed (by A. J. B.) to extend their body when the shell is knocked. *Pomacea urceus* aestivates next to the ground surface where conditions in the dry soil are probably not anaerobic. The soil cracks around the shell make oxygen more available to the snail. Further, a good oxygen supply is assumed necessary for the developing eggs in the brood chamber beneath the female. In his discussion of anaerobic habitats, von Brand (1946) points out that in most soils the oxygen concentration is sufficient for aerobic respiration. However, after rain when the soil is wet, the exchange of gases with the air is reduced and the ground oxygen can be rapidly depleted. Anaerobic conditions for aestivating *Pomacea urceus* are most likely to occur at the beginning and at the end of the dry season. It would not be surprising if these snails use pulmonary respiration via their siphon when they first burrow into the mud at the water's edge. At the end of the dry season the first heavy rains could fill the burrows and cut off the oxygen supply, but burrows are superficial and anaerobic conditions could involve only a few hours. Also, these snails become active in the presence of water at the end of the dry season. This is particularly rapid in spat since only minutes are necessary for full activity.

Attention has been drawn to the annual sequence of rainy and dry seasons of the Venezuelan plains and to the terrestrial and aquatic phases in the life cycle of these snails. The ampullariid gastropods are doubly adapted in terms of their "amphibious" respiratory structures. The presence of unlimited water or its relative absence provides different conditions for temperature experience and activity. The behavioral adaptation of burrowing and the subsequent inactivity (aestivation with lowered metabolism) are of advantage for survival during the dry season. Further, uricotellism and tolerance of dehydration are both adaptive under arid conditions. The existence of wet and dry seasons stress the importance of adaptations for an amphibious way of life.

We would like to thank Dr. T. Pain for having examined specimens of this study and for having identified these snails as typical *Pomacea urceus* (Müller); Professor Rafael Martinez for making equipment available and for informative discussions about the natural history of snails in the Llanos region of Venezuela; Eduardo Miranda and Oswaldo Travieso for their assistance in collecting snails; the other students who aided in the recording of data; and Kathleen A. Burky for assistance in field collecting and during the preparation of this paper.

SUMMARY

1. It has been demonstrated that adult snails generally regulate their body temperature below 41° C under experimental conditions and that their upper lethal temperature is between 40 and 45° C.

2. Field data indicate that under natural conditions adult body heat is transfered to the ground of the aestivation burrow by conduction and that this heat is at least in part dissipated by evaporative loss of soil moisture.

3. Under experimental conditions snails can survive for an aestivation period of four times the normal length and with a loss of 62% of their tissue weight. This level of experimental water loss would be inadequate as the only agent of temperature regulation under field conditions but could be a supplement to heat transfer to both soil and air as well as evaporative cooling afforded by the ground.

4. Aestivating adults can survive many days of direct exposure to the tropical sun (out of burrow) while juveniles are dead within two hours or less.

5. The metabolism of aestivation is aerobic with oxygen consumption about one fifth that of active snails.

6. Females provide protection from high temperatures and from water loss for eggs and spat during the dry season. The adaptiveness of superficial aestivation burrows is discussed in relation to the needs of aerobic metabolism for adults and developing eggs.

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REGENERATION OF THE PROBOSCIS OF MURICID GASTROPODS AFTER AMPUTATION, WITH EMPHASIS ON THE RADULA AND CARTILAGES

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Boring of prey shells by muricid gastropods involves the close interaction of the proboscis, propodium, and the accessory boring organ (ABO) in a predictable cycle which repeats itself continuously throughout the process of boring of each borehole. Although the radula serves only a minor part in shaping the borehole, it appears that both the radula and the ABO are necessary to effect normal penetration (Carriker and Van Zandt, 1972). Whether this is so could be tested by inactivation of one and then the other organ in different individuals and noting the effect on penetration. The first objective of the present study was to examine the effect of removal of the proboscis on boring.

In 1957 an adult *Urosalpinx cinerea* in our laboratory caught its proboscis between the glass and shell of an oyster model (Carriker and Van Zandt, 1972), and after tugging for some time to free itself, tore away the proboscis leaving the distal third wedged in the model. The snail recovered and resumed boring of prey in 20 days. The previous year Demoran and Gunter (1956) experimentally amputated the distal portion of the proboscis of several thaisid boring snails, *Thais haemastoma*, and reported that these gastropods regenerated the proboscis within three weeks provided the organ had been cut off cleanly. From time to time the senior author has dissected large adult *Urosalpinx cinerea* collected in the field in which the anterior portion of the proboscis, though abnormally small, externally appeared morphologically normal. The small size of the proboscis tip indicated that these snails had accidently lost and regenerated the proboscis. These several observations suggest that regeneration of the complex buccal mass in *Urosalpinx cinerea* (Carriker, 1943) and other predatory marine gastropods may occur normally in nature. The second objective of this investigation was thus to determine how commonly and at what rate regeneration occurs in the laboratory after experimental proboscisectomy. A preliminary summary of the results of these studies was published by Carriker (1959, 1961). The radula, being a relatively hard structure (Carriker and Van Zandt, 1972) provides a readily measurable parameter for quantitative determination of regeneration.

During the past several decades regeneration of cartilages has been investigated almost exclusively in vertebrate animals. Recently, however, following a long period of neglect, invertebrate cartilages again came under serious study (Person and Philpott, 1969; Mathews, 1968). As part of a survey of the nature and properties of cartilages in various invertebrate phyla, Person became interested in their regenerative capacity. In the reports by Demoran and Gunter (1956) and Carriker (1961) neither specific mention nor details were given concerning regeneration of the odontophoral cartilages which were removed by proboscisectomy. The third objective of the investigation was therefore to ascertain whether complete histological regeneration of these structures occurs following proboscisectomy.

MATERIALS AND METHODS

Healthy well fed individuals of *Urosalpinx cinerea* (Say), *Urosalpinx cinerea follyensis* Baker, and *Eupleura caudata etterae* Baker (Family Muricidae, Class Prosobranchia) were employed in the investigation. Three separate experiments were run. The first was carried out during the summer of 1958 at the University of North Carolina Institute of Fisheries Research, Morehead City, North Carolina, and employed *Urosalpinx cinerea* from the local sounds of North Carolina, and *Eupleura caudata etterae* from Chincoteague Bay, Virginia. The second (1965) and third (1970) experiments were conducted at the Marine Biological Laboratory and employed only *Urosalpinx cinerea follyensis* from Wachapreague, Virginia.

Snails were shipped airmail and maintained in the laboratory in rapidly running seawater. They were fed oysters and acclimatized readily to laboratory conditions. At the Institute of Fisheries Research the temperature of the running seawater during the experiment ranged between 23.5 and 33.0° C, and the salinity between 27 and 32‰. At the Marine Biological Laboratory in 1965 the temperature of the running seawater varied between 18.0 and 22.5° C, the salinity between 31.9 and 32.0‰ and the pH between 8.09 and 8.11. In 1970 the temperature fluctuated between 20.3 and 23.8° C, the salinity between 31.3 and 31.5‰ and the pH between 8.03 and 8.14. During the daytime snails were illuminated by daylight coming through the laboratory windows, and during the early part of the evening by standard overhead artificial laboratory light.

The technique devised for proboscisectomy was developed in the summer of 1957, and was as follows: Into a tray of running seawater we placed a kitchen-type plastic dish, approximately 7.5 by 7.5 by 10 cm, whose sides had been perforated many times to allow ready passage of seawater. A plastic screen, pore size approximately 1 mm, had been cemented into the dish to provide an upright diagonal divider, creating two triangular compartments. In the upstream compartment we placed a freshly shucked live oyster with its flesh against the screen; in the downstream compartment we placed four to six snails. After several hours one or more snails were attracted to the oyster, and after mounting the screen and inserting their proboscides through the screen, they began to feed on the oyster. After the snails had been feeding for a few hours, they could be pulled gently away from the screen without detaching the everted proboscis from the oyster, thereby extending the proboscis further. The considerable length of the proboscis when fully everted, about the same as the height of the snail shell, facilitated the operation. An iris scissors was then carefully inserted between the screen and the snail, and the proboscis was cut quickly and cleanly close to its base, removing with it the buccal mass and more or less of the radular sac depending on the plane of amputation (Fig. 1) and the degree of extension of the sac. The snails were then allowed to recuperate undisturbed in running seawater. Excision of the buccal mass, radula, supporting odontophoral cartilages, and other structures of the proboscis were verified by examination of the amputated proboscis with a dissecting microscope. For the details of normal morphology of the adult proboscis, see Carriker (1943). No mortality resulted from the proboscisectomies, and all snails recovered rapidly.

The 1958 experiment was designed to determine (a) the number of days after proboscisectomy when boring would resume, and (b) the rate of anatomical regeneration of the proboscis after proboscisectomy. A total of 64 snails was successfully proboscisectomized, 32 snails for (a) and 32 for (b) (see below). Each of (a) and (b) included 16 *Urosalpinx cinerea* (8 males and 8 females, 4 medium and 4 large individuals of each sex) and 16 *Eupleura caudata etterae* (8 males and 8 females, 4 medium and 4 large individuals of each sex). The *Urosalpinx cinerea* ranged in shell height from 16.5 to 25.9 mm, and the *Eupleura caudata etterae* from 19.6 to 34.2 mm.

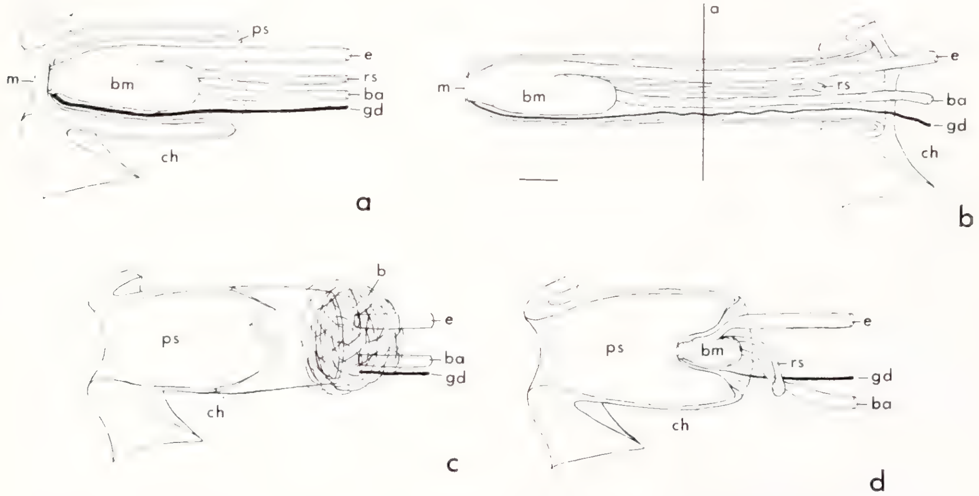

FIGURE 1. Diagrams illustrating the gross anatomy, amputation, and regeneration of the proboscis of *Urosalpinx cinerea,* shell height approximately 25 mm: (a) median plane of the proboscis retracted within the proboscis sac in the cephalic hemocoel; (b) line a, approximate plane at which proboscis amputated; (c) blastema joining the proboscis stump and ends of esophagus, buccal artery, and accessory salivary gland duct; (d) regenerated proboscis tip, buccal mass, and radular sac; a, plane of amputation; b, blastema; ba, buccal artery; bm, buccal mass; ch, cephalic hemocoel; e, esophagus; gd, accessory salivary gland duct; m, mouth; ps, proboscis sac; rs, radular sac; scale bar, 1 mm.

In (a), immediately after proboscisectomy, snails were placed in small translucent perforated plastic dishes in running seawater filtered through coarse sand and shell fragments, each snail isolated with a live oyster 5–7 cm long and cleaned of encrustations. After remaining quietly in a corner of the dish for a day or so, each snail crawled about, and in time was attracted to the oyster and mounted it. Thereafter each day with a needle we gently pushed the midanterior portion of the propodium of the snail back to a point immediately under the ABO. If boring had begun, the initial borehole was clearly evident, the snail was sacrificed, and the

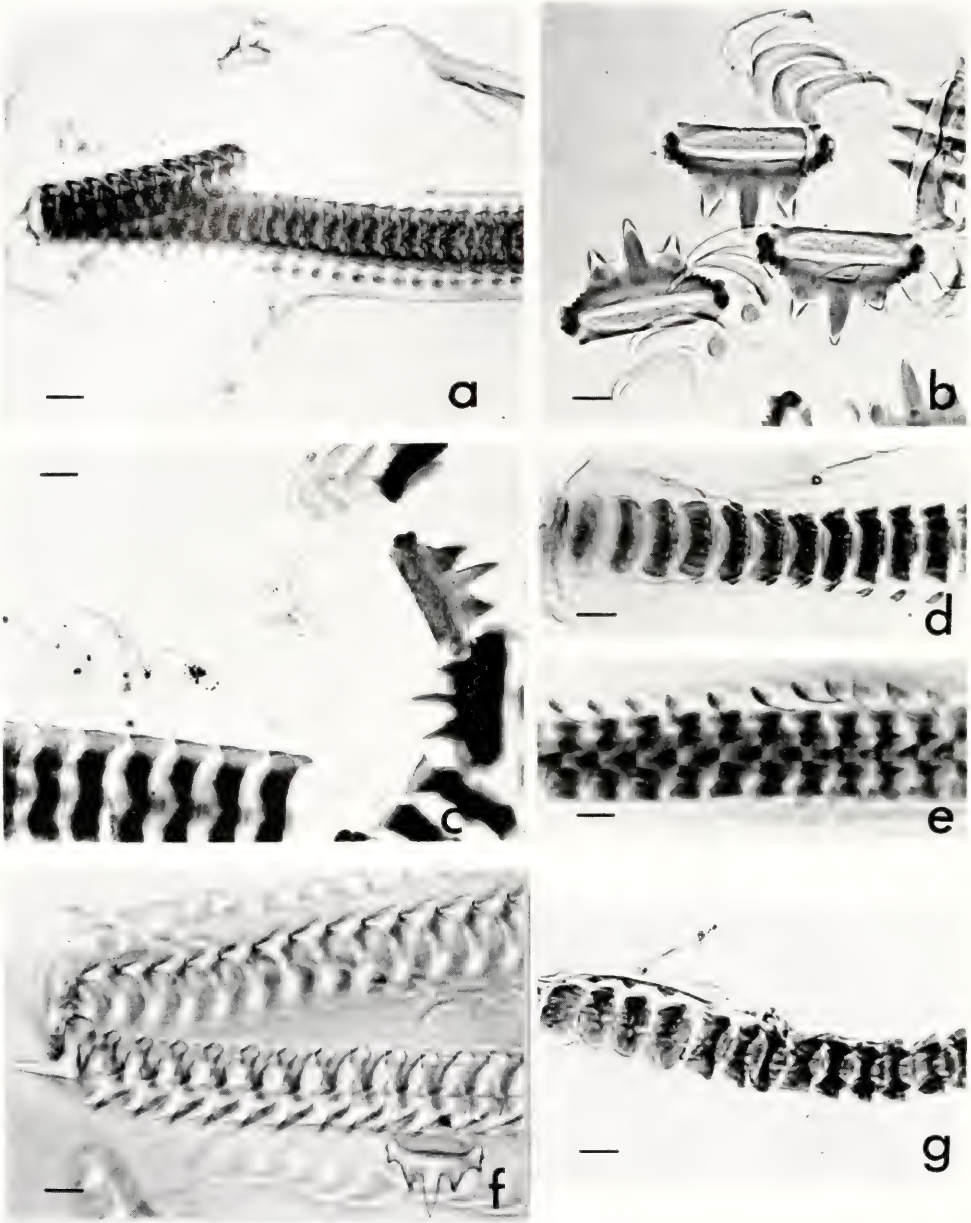

FIGURE 2. a–e: light micrographs of radulae of *Urosalpinx cinerea:* (a) anterior end of normal radula of adult snail on subradular membrane; scale bar, 35 μ; (b) marginal (single cusped) and rachidian (tricusped) teeth of normal radula of adult snail; scale bar, 20 μ; (c) parts of regenerated radula 100 days after snail tore off the anterior third of the proboscis which was wedged in a laboratory device, snail shell height 25 mm; scale bar, 20 μ; (d) anterior portion of regenerating radula 12 days after proboscisectomy; scale bar, 20 μ; (e) posterior portion of same radula as in (d); scale bar, 20 μ. f–g: light micrographs of radulae of *Eupleura*

proboscis was examined for extent of regeneration. If boring had not begun, the snail and oyster were returned to the dish. Oysters were changed weekly with individuals freshly collected in the field. Oysters held in captivity, even in running seawater, tend rapidly to lose their attractiveness after a few days (Carriker and Van Zandt, 1972), possibly because of decreased food.

In (b) proboscisectomized snails were likewise placed in dishes in running filtered seawater, but without oysters. Eight snails (a large and a small male, and a large and a small female of each species) were sacrificed on the 4th, 8th, 12th, and 16th day after amputation, and the regenerating proboscides were examined for degree of anatomical regeneration.

At the time of sacrifice the shell of the recuperating snail was cracked open by a blow with a hammer, and the animal was removed. The regenerating proboscis was then excised from the cephalic hemocoel of the snail, opened under a dissecting microscope, held in position with minute dissecting pins on the exposed surface of a rubber eraser embedded in wax in a dissecting pan, and examined under seawater. Tissues were stained with Little's methylene blue as the dissection progressed. Radulae, if present, were freed of soft tissue in 10% KOH, washed, stained in 1% aqueous chromic acid, dehydrated in alcohols, and mounted flat in Euparol to facilitate examination of the teeth. Because of the tendency of radulae to curl, some of them turned on the side during mounting. Study and measurement of the teeth was done with an ocular micrometer in a compound microscope.

The 1965 experiment was designed to provide regenerating proboscides at intervals of 4, 8, 12, and 20 days after proboscisectomy for histological examination. Thirteen individuals of *Urosalpinx cinerea follyensis*, ranging in shell height from 25 to 43 mm, were utilized, three specimens each for the 4, 8, and 12 day periods, and four for the 20 day period. Regenerating proboscides were everted from the cephalic hemocoel (except in the earliest cases where the proboscis was too small to permit orientation), excised at the base, fixed in cold Bouin's, dehydrated in ethyl alcohols, and embedded in paraffin. Sections were cut 7 μ thick on a standard microtome parallel to the long (anterior-posterior) axis. One half of the sections in each series was stained with Lillie's modification of Masson's trichrome stain, and alternate serial sections were stained with astra blue, Weigert's iron hematoxylin, or Mallory-Heidenhain's stain.

In the 1970 experiment, designed to study the histology and cytology of the regenerating cartilages of the buccal mass, recuperating *Urosalpinx cinerea follyensis* (ranging in shell height approximately from 27 to 38 mm) were sacrificed at time intervals of 3, 7, 11, and 19 days post-proboscisectomy. A minimum of four snails was used in each group. The regenerating proboscides were removed under a dissecting microscope, and half were placed in Bouin's fixative for paraffin embedding, and the remainder were frozen immediately on a quick-freeze stage. Frozen sections 8 μ in thickness were prepared in a Slee freezing microtome for toluidine-blue staining at pH 3. The paraffin embedded sections (5 μ thick) were stained with Wright's nuclear stain, astra blue, or Mallory triple stain.

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caudata etterae; (f) anterior end of normal radula on subradular membrane, snail shell height 25 mm; scale bar, 23 μ; (g) regenerating radula 8 days after experimental proboscisectomy, snail shell height 28 mm, anterior portion of the radula to the left, posterior portion to the right; scale bar, 23 μ.

RESULTS

Regenerative changes and rates

In 1958 all 32 individuals of *Urosalpinx cinerea* and *Eupleura caudata etterae* which were allowed to resume boring (experiment a), had regenerated the proboscis, and this organ, though proportionately small, appeared normal under the dissecting microscope. Traces of pink color (probably myoglobin) appeared in the musculature of the buccal mass by the 15th day, and by the 20th day this became more intense. The normal buccal mass in the adult snail is brightly colored. The 32 individuals of both species which were sacrificed at four day intervals after proboscisectomy (experiment b), had all begun regeneration of the proboscis, the degree of development increasing with time past amputation. By the 4th day, at least a filmy cap of loose tissue covered the stump of the proboscis and bound the amputated ends of the esophagus, buccal artery, ducts of the salivary glands, and other tissues to it (Fig. 1c). In two snails (one of each species) the external form of the minute radular sac was already clearly evident. Between the 8th and 12th days after the operation, the four individuals of both species had formed minute normal proboscides and radulae (Fig. 1d), and by the 16th day the radulae had approximately doubled in length.

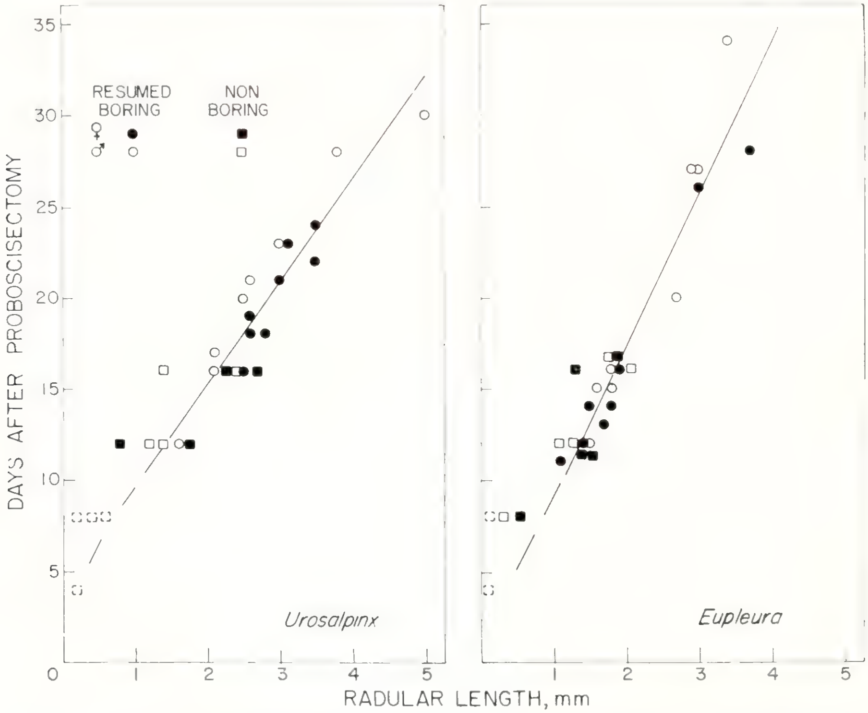

FIGURE 3. Extent of regeneration of radulae of male and female *Urosalpinx cinerea* and *Eupleura caudata etterae* 4, 8, 12, and 16 days after proboscisectomy ("non boring"), and at resumption of boring after proboscisectomy ("resumed boring").

In two snails, where amputation of the proboscis had taken place at the level of the radular sac, a part of the large original radula, now devoid of its sac but otherwise intact and showing no sign of dissolution, was found in the base of the regenerating proboscis.

In the 8th and 12th day regenerating radulae, the earliest rachidian and marginal teeth were small and slightly misshapen (Fig. 2). In one radula the early midrachidian cusp was forked for several initial transverse rows, then in one row changed from the forked to the normal unicuspid condition. Earliest rachidial teeth were separated from each other more than were normal teeth, and even in very small radulae were heavily worn by abrasion, suggesting that the snail starts rasping soon after the teeth are first formed. Teeth increased rapidly in size along the long axis of the radula with time, and successive rows achieved the normal form quickly, the rachidian teeth first, and some time later, the marginal teeth. Earliest regenerating marginal teeth started as short, weak, thread-like structures.

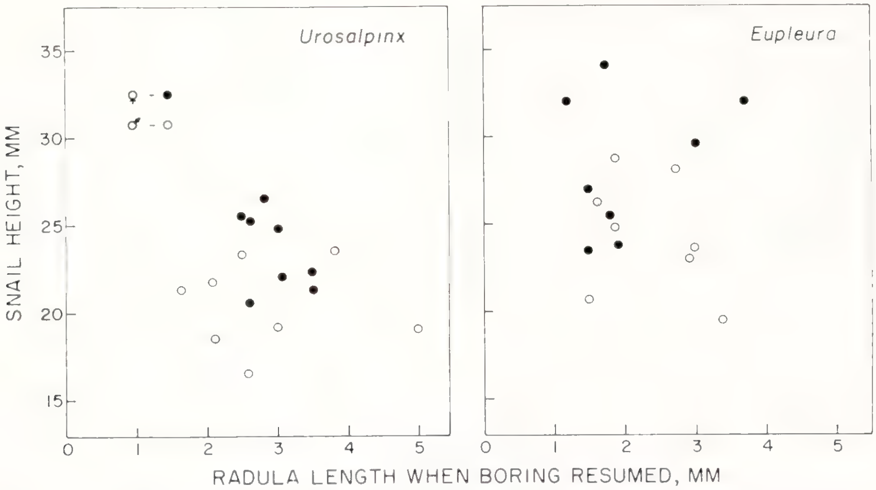

FIGURE 4. Length of radulae and shell height of male and female *Urosalpinx cinerea* and *Eupleura caudata etterae* at resumption of boring.

The approximate rate of regeneration of radulae in *Urosalpinx cinerea* and *Eupleura caudata etterae* is plotted in Figure 3. Initial regeneration was a little faster in *Eupleura caudata etterae* than in *Urosalpinx cinerea*, but the length of the radula increased at a faster rate in some *Urosalpinx cinerea*. There were noticeable differences in the growth rate of radulae among individual snails, but no consistent differences between the growth rate of radulae of males and females.

Although the rate of increase of radulae of both species was surprisingly rapid and relatively uniform, the time of onset of shell penetration of prey varied from 11 to 34 days (Fig. 3). No obvious differences in the onset of boring by males and females was evident. Likewise there was no association between the length of the radula and the height of the snail shell when boring was resumed (Fig. 4).

Measurement with an ocular micrometer of the widest portion of the base of rachidian teeth at the anterior (oldest) and posterior (newest) ends of (a) normal radulae, (b) regenerating radulae of snails allowed to resume boring after proboscisectomy, and (c) regenerating radulae of snails sacrificed at intervals after amputation, clearly demonstrated the rapid rate of enlargement of teeth with time after proboscisectomy (Fig. 5). However, there was noticeable variation in size between old and new rachidian teeth in some of the normal adult snails of both species: in some, newest teeth were smaller than old ones; in others, they were larger; and in still others, the radula was constant in size throughout its length. Rate of widening of rachidian teeth was maximal in the earliest stages of regeneration of the proboscis (Fig. 5).

Examination of incomplete boreholes excavated by snails which were allowed to bore after regeneration of the amputated proboscis, disclosed that 12 of the holes made by *Urosalpinx cinerea,* and 13 by *Eupleura caudata etterea,* were normal as to size and shape (see Carriker and Yochelson, 1968), and 4 and 3, respectively, were slightly abnormal only in shape. The abnormality, however, appeared to be caused more by the uneveness and irregularity of the growth rings in the oyster shell than by malfunctioning of the radula.

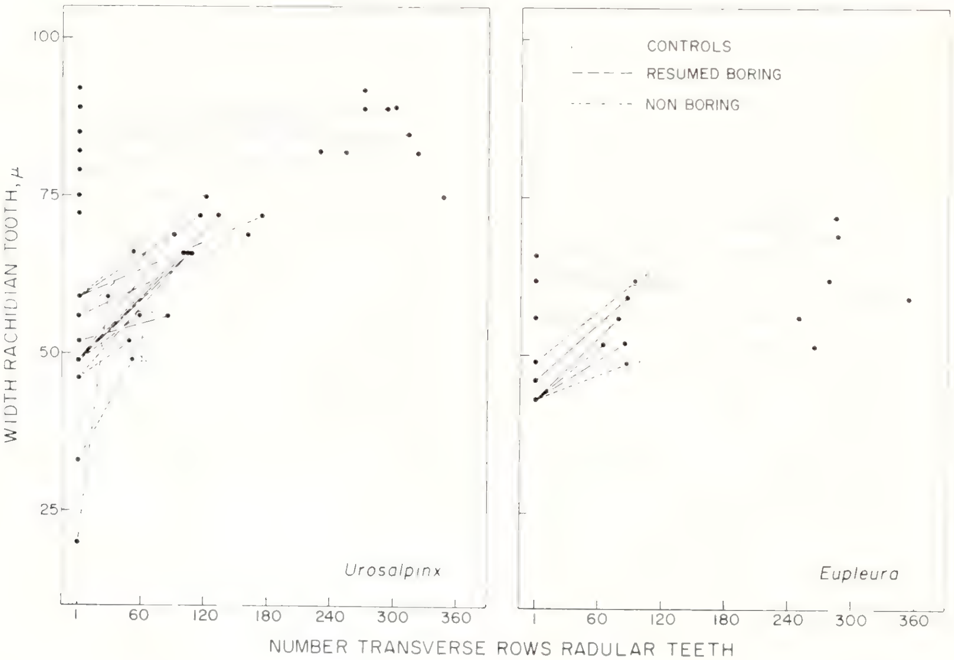

FIGURE 5. Change in width of the base of rachidian teeth of *Urosalpinx cinerea* and *Eupleura caudata etterae* from the anterior (old portion) to the posterior (new portion) of the radulae: in normal snails ("controls"), in snails whose proboscides were amputated and then allowed to regenerate and resume boring ("resumed boring"), and in snails whose proboscides were amputated and then the snails were sacrificed at 4, 8, 12, and 16 days after amputation ("non boring"). Numbers beside points refer to the number of days after proboscisectomy when snails were sacrificed. Teeth were counted from the anterior to the posterior of the radulae.

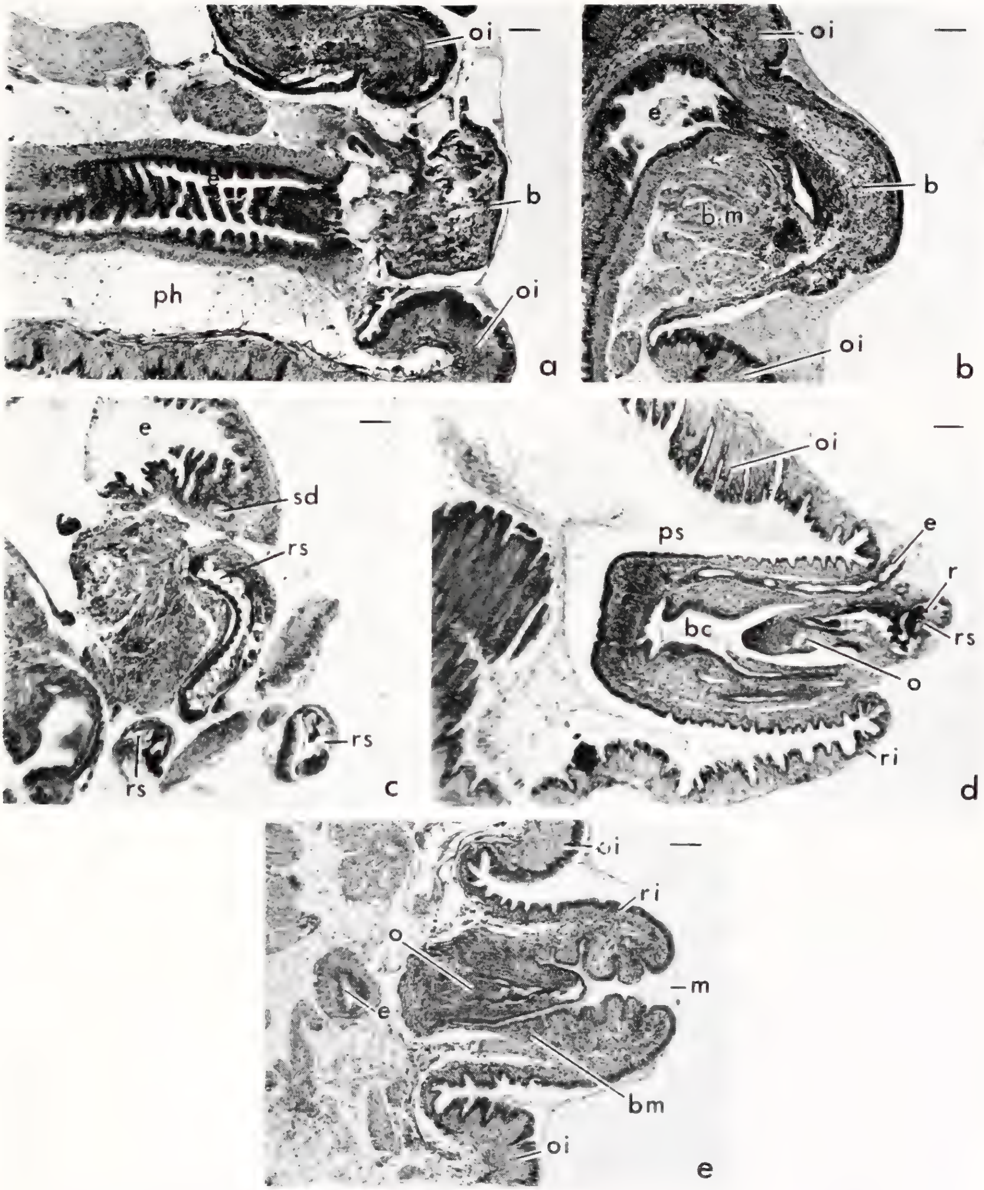

FIGURE 6. Representative stages in the regeneration of the proboscis of *Urosalpinx cinerea follyensis* following proboscisectomy. Snails were sacrificed 4, 8, 12, and 20 days after amputation; light micrographs; Astra blue, Weigert's iron hematoxylin, and Mallory-Heidenhain's stains: (a) initial stage illustrating early blastema, 4 days after amputation; scale bar, 40 μ; (b) early stage, 12 days after amputation; scale bar, 85 μ; (c) intermediate stage showing radula and radular sac, 8 days after amputation; scale bar, 100 μ; (d) new proboscis, withdrawn in proboscis sac, 20 days after amputation; scale bar, 100 μ; (e) new proboscis, everted, 20 days after amputation; scale bar, 100 μ; bc, buccal cavity; b, blastema; c, cap cell; bm, buccal musculature; ct, cartilage, e, esophagus, m, mouth; o, odontophore; od, odontophoral musculature; oi, old proboscis integument; ph, proboscis hemocoel; ps, proboscis sac; ri, regenerating proboscis integument; rs, radular sac; r, radula, sd, salivary gland duct.

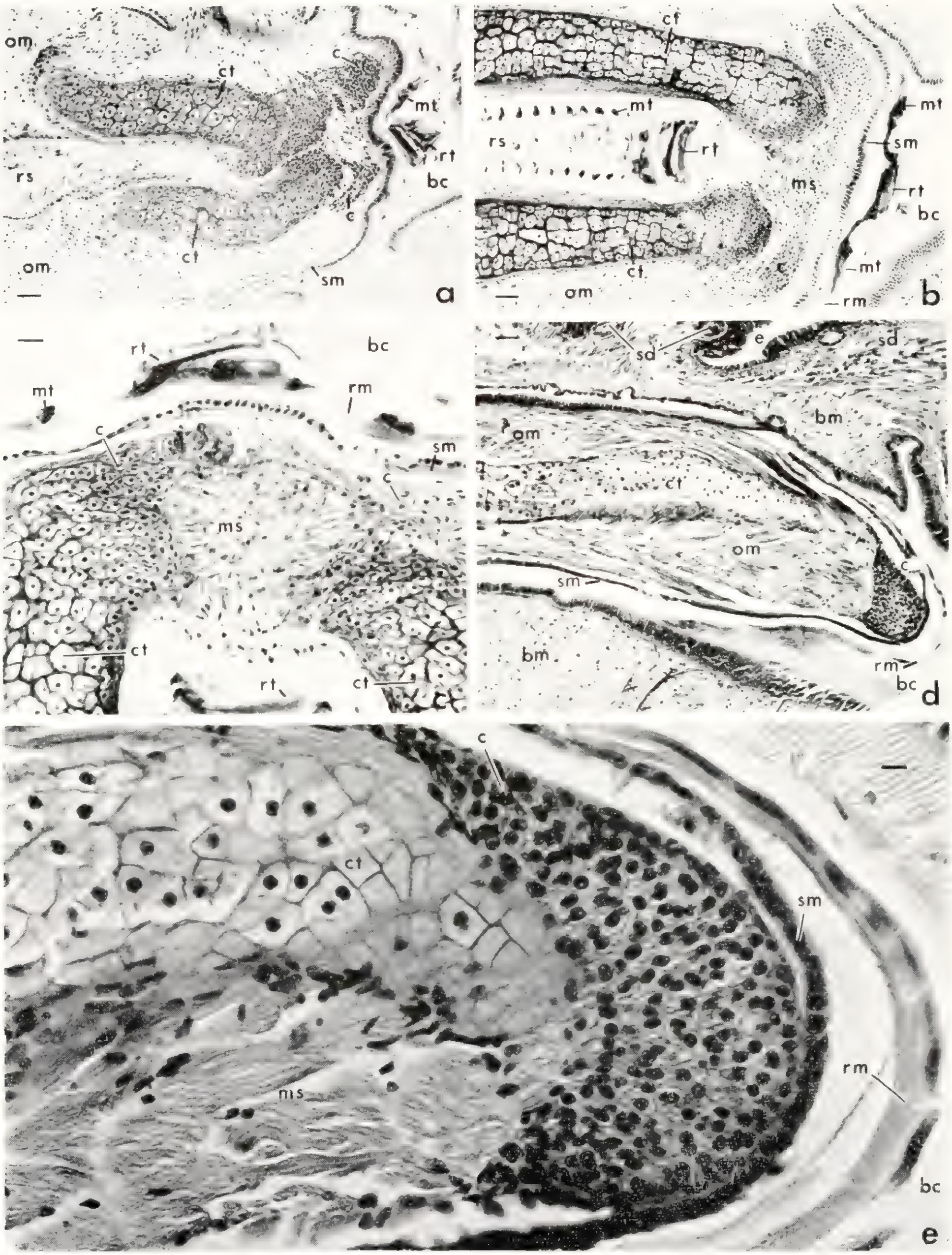
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FIGURE 7. Representative stages in normal and regenerating odontophores of *Urosalpinx cinerea follyensis*: (a) frontal section of odontophoral cartilages in nonoperated (control) snail; frozen section, toluidine blue stain; scale bar, 85 μ; (b) frontal section of regenerating odontophoral cartilages in a snail 11 days after proboscisectomy, the section is deeper into the cartilage than in (a), frozen section, toluidine blue stain; scale bar, 85 μ; (c) frontal section of anterior

Histology of regenerating proboscides

Although all 13 proboscisectomized individuals of *Urosalpinx cinerea follyensis* in the 1965 experiment commenced regeneration of the proboscis tip, the rate of regeneration within each time interval varied noticeably, in contrast to the relatively uniform results obtained in the 1958 experiment. Whether this was due to subspecific differences, or to variation in the level of amputation of the proboscis, is not known.

General trends in regeneration of the organs of the proboscis of *Urosalpinx cinerea follyensis* are illustrated in Figure 6. Initially a loose mass of cells, including numerous amebocytes, formed over the cut end of the proboscis and joined this to the cut ends of the esophagus, buccal artery, and other tissues (Fig. 6a). The epithelium and muscular layers of the esophagus then grew forward into the blastema, and the integument of the proboscis extended over the blastema as a thin epidermis one cell thick with mucous cells (Fig. 6b). Simultaneously muscle fibers appeared within the blastema. From this mass arose the integument and musculature of the new proboscis tip, the buccal mass and its musculature, the forward end of the esophagus, salivary and accessory salivary gland ducts, arteries, and nerves, the odontophore, cartilages, radular sac, radula, and cuticular lining of the buccal cavity (Figs. 6c–e, 7d). Odontophoral cartilages, radular sac, and radula were histologically distinguishable by the 8th day after proboscisectomy.

Cytology of regenerating odontophoral cartilages

In the 1970 experiment regenerating radulae and odontophoral cartilages of *Urosalpinx cinerea follyensis* were well advanced by the 7th and 11th days, respectively, after proboscisectomy.

For reference purposes, we will first illustrate the histology of the odontophore of a normal nonoperated *Urosalpinx cinerea follyensis* (Fig. 7a). The cartilages were sectioned in a dorsal peripheral plane of the odontophore, and lie surrounded by the odontophoral musculature (om). The radular sac (rs) emerges posteriorly between the cartilages, and rachidian teeth (rt) are visible on the anterior tip of the odontophore in the buccal cavity (bc). At its anterior end each cartilage possesses a cap of cells (c) which as will be seen, is of complex morphology, and appears closely related to tissues of both the odontophoral cartilages and the surrounding muscle. Nuclei of the cartilage cells stained orthochromatically (blue), whereas the cytoplasm and intercellular matrix of these cells were primarily (but not entirely) strongly metachromatic (pink, purple). Muscle fibers, their nuclei, and

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ends of regenerating odontophoral cartilages in a snail 11 days after proboscisectomy. Note details of cap cells and both muscle and cartilage cells issuing from them; frozen section, toluidine blue stain; scale bar, 35 μ; (d) sagittal section through one of the odontophoral cartilages in a snail 20 days after proboscisectomy, illustrating the relationship between the cap, muscle, and cartilage cells; Bouin's fixative, Wright astra blue, and Mallory's stains; scale bar, 85 μ; (e) sagittal section through anterior tip of odontophoral cartilage in a snail 20 days after proboscisectomy, serial section from same specimen as in (d); Bouin's fixative, astra blue, Weigert's iron hematoxylin, and Mallory-Heidenhain's stains; scale bar, 10 μ; bc, buccal cavity, bm, buccal musculature; c, cap cells; ct, cartilage; e, esophagus; ms, muscle cells; mt, marginal teeth; om, odontophoral musculature; r, radula; rm, radular membrane; rs, radular sac; rt, rachidian teeth; sd, salivary gland duct; sm, subradular membrane.

the nuclei of the cap cells were also orthochromatic, while the cytoplasm and intercellular substance of the cap cells likewise exhibited metachromasia.

Regeneration of the cartilages will be described from representative sections of the proboscis from snails 11, 19, and 20 days after proboscisectomy. Figure 7b is a photomicrograph of a frontal section of the odontophore of an 11 day post-proboscisectomized snail. The plane of the section was deeper into the cartilages than was the case in the specimen shown in Figure 7a. It is evident that by the 11th day considerable regeneration of cartilage, radula, and associated tissues had taken place. The distribution of metachromasia and orthochromasia in the tissues appeared similar to that described for the section of Figure 7a, but the metachromasia was more intense. Toward the anterior end of each cartilage in Figure 7b, the cells become smaller in size, and eventually merge with still smaller epitheloid cells which form the cap referred to earlier in the nonoperated snail. At the periphery of the cap, muscle fibers (ms) are in close proximity and appear to interweave with the cap cells (c). This is more clearly illustrated in Figure 7c, a higher magnification of the cap region in a serial section twice removed from that shown in Figure 7b. Both cartilage cells (ct) and muscle cells (ms) blend imperceptibly with the cap cells (Fig. 7c), giving the impression of a blastema-like structure reminiscent of that seen in vertebrate limb regeneration. This impression was strengthened by Figure 7d and 7e which illustrate sagittal sections of regenerated odontophores 20 days after proboscisectomy. At low magnification (Fig. 7d) the cap appears as a tightly packed, rapidly dividing mass of cells from which both cartilage and muscle are forming. At a higher magnification (Fig. 7e) in a serial section twice removed from that shown in Figure 7d, the imperceptible blending of both cartilage and muscle cells with those of the cap is unmistakable, and the resemblance to a vertebrate blastema is reinforced.

DISCUSSION

The ability of muricid gastropods to penetrate the shell of prey allows them, protected by their own shell and for a time by the valves of the prey, to feed on otherwise generally inaccessible organisms often much larger than themselves. After penetration of the shell and while feeding on gaping moribund oysters, however, snails risk loss of the proboscis in two ways: (a) amputation by small crabs and fish when the proboscis is extended into the mantle cavity through the borehole, and (b) pinching and subsequent loss while the proboscis is inserted between the valves. Amputation by both means may occur in nature, though how frequently is not known. Valvular motion of normal live prey inhibits boring between the edges of the valves (Carriker and Van Zandt, 1972), so the danger in (b) is from a prey which after gaping widely for a time suddenly clamps shut, irritated by scavengers feeding on its tissues.

The present studies demonstrated that regeneration of the proboscis takes place in a remarkably short time, and proboscisectomized snails, even the occasional ones in which the amputation is ragged, recover. Rapid functional replacement of the feeding organ insures survival, and an accident which otherwise might have had disastrous consequences, is only a passing inconvenience. The unusual capacity of mollusks to regenerate lost parts has been known for a long time (Hyman, 1967), but the rapid regeneration of so complicated an organ system as the prosobranch proboscis has not been reported prior to this investigation and the paper by Demoran and Gunter (1956). Isarankura and Runham (1968), by marking the radulae of live pulmonates and prosobranchs (including the muricid, *Thais lapillus*) by various techniques, determined that the rate of replacement (forward movement) of the radula over the odontophore is continuous. The present studies support their findings.

The effect of removal of the proboscis on the capacity of boring snails to penetrate the shell of oysters was demonstrated by individuals of *Urosalpinx* and *Eupleura* which were allowed to resume boring after proboscisectomy. In every case, boring was initiated only after the radula and associated structures had developed normally, anatomically and histologically. Earliest regenerating teeth increased most rapidly in size. Isarankura and Runham (1968) also reported a very rapid rate of replacement of the radula in newly hatched prosobranchs and pulmonates, followed by a steady decrease in replacement rate. Although the rate of increase in length of radulae of *Urosalpinx* and *Eupleura* soon after proboscisectomy was rapid and unform, the time of onset of boring of prey ranged over a period of 23 days in different individuals. Thus development of a given length of radula did not trigger penetration, and the initiation of boring was stimulated by other factors, possibly attractiveness of prey, or the physiological and behavioral condition of the snails, or both. The noticeable variation in size between old and new rachidian teeth in some adult, nonoperated individuals of both *Urosalpinx* and *Eupleura* was unexpected. Changes, when they occurred, were gradual down the length of the radula, so it is difficult to ascribe them to nutritional causes. Resumption of boring only after the radula and associated structures appeared anatomically normal suggests that the redula is an essential component of the mechanism of shell penetration. Furthermore, the capacity of adult snails with small newly regenerating radulae to excavate boreholes of a shape and size similar to those of normal adult snails is further evidence that the shape and size of the borehole are the products primarily of chemical activity of the accessory boring organ rather than of the radula (Carriker and Van Zandt, 1972).

Regenerating odontophoral cartilages of *Urosalpinx* are strikingly similar in histological appearance to the regenerating limbs of vertebrates, as seen, for example, in the salamander (Butler, 1933; Kiortsis and Trampush, 1965; Thornton, 1968). In both instances the regenerative process is considerably dependent upon a unique cell aggregate, the blastema of vertebrates, and its analogue, which we have called the cap cells, of *Urosalpinx*. Although little is known of the chemistry of the cartilage of these snails, its strong metachromatic staining with toluidine blue at pH 3 indicates the probable presence in the tissue of macromolecular anionic polysaccharides. In *Busycon,* a genus of predatory marine snails, Lash and Whitehouse (1960) reported the presence in the odontophoral cartilage of a nonaminated polyglucose sulfate. Person and Philpott (1963) have also shown that collagen is present in the odontophoral cartilage of *Busycon.* Although at the present time no chemical or ultrastructural data dealing with the odontophoral cartilage of *Urosalpinx* are available, it is likely that some form of anionic polysaccharide and also collagen will be found in its tissue. In view of these findings, and of the relatively rapid regeneration of muricid odontophoral cartilage, we suggest that these and other gastropod families may prove useful for the study of cartilage and skeletal regeneration.

The boring habit and the capacity for rapid regeneration of the proboscis provide unusual advantages in procurement of food, and these perhaps account in large part for the biological success of such muricid species as *Urosalpinx cinerea* and *Eupleura caudata* and their significance as major predators of commercial oysters.

John W. Blake assisted in the investigation in 1958, Barry Martin in 1965, and Robert Lipson in 1970. Photographs resulting from the 1958 and 1965 studies were taken by Peter J. Oldham. The live specimens of *Eupleura caudata etterae* used in 1958 were supplied by Michael Castagna, Thomas Carter, and George Griffith from Chincoteague Bay, Maryland-Virginia; the live specimens of *Urosalpinx cinerea follyensis* employed in 1965 and 1970 were supplied by Michael Castagna from Wachapreague, Virginia.

The research in 1958 was supported in part by a grant from the U. S. Fish and Wildlife Service; that in 1965 by Public Health Service Research Grant DE 01870 from the National Institute of Health; and that in 1970 jointly by Public Health Service Research Grant DE 01870 and the Veterans Administration.

Acknowledgment is gratefully made for the many courtesies and generous assistance which made this study possible. Systematics-Ecology Program Contribution No. 264.

SUMMARY

1. All individuals of *Urosalpinx cinerea, Urosalpinx cinerea follyensis,* and *Eupleura caudata etterae* from which the proboscis was removed, recovered, and fully regenerated the proboscis. By the 4th day after proboscisectomy a blastema of loose tissue bound the amputated ends of the esophagus, buccal artery, ducts of the salivary glands, and the other tissues to the stump of the proboscis. Between the 8th and 12th days after the operation, snails had formed minute proboscides and radulae. Onset of boring of shell by regenerating snails varied from 11 to 34 days, and took place only after the radula and associated structures were developed and functional. The radula is thus an essential component of the mechanism of shell penetration. Formation of a given length of radula did not trigger penetration; other unexplained factors appear to be responsible.

2. Although earliest regenerating rachidian and marginal teeth were small and misshapen, increase in size and normalization of form was rapid. In time regeneration of proboscides was complete, and they resembled normal proboscides anatomically, histologically, and functionally. Earliest teeth were worn by abrasion, indicating that snails began rasping soon after the teeth and odontophore were formed. Boreholes excavated by snails with small newly regenerating radulae generally corresponded in form and size to those bored by normal snails; this is evidence that the shape and size of the borehole are the products of chemical activity of the accessory boring organ rather than the radula.

3. Histologically the organization of the regenerating odontophoral cartilages and associated musculature and other tissues was similar to that seen in regenerating vertebrate limbs. In both cases the regenerative process is dependent upon a unique cell aggregate (the blastema of vertebrates, and its analogue in muricid

snails), a cap of cells organized at the regenerating tip of the amputated structure.

4. The boring habit and rapid regeneration of the proboscis are distinct assets in procurement of food, and perhaps account in part for the biological success of *Urosalpinx cinerea* and *Eupleura caudata* and their significance as major predators of commercial oysters.

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LATITUDINAL EFFECTS ON METABOLIC RATES IN THE CRICKET FROG, *ACRIS CREPITANS:* ACUTELY MEASURED RATES IN SUMMER FROGS

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If a poikilothermic animal could not compensate for temperature in metabolic activity, the rates of chemical reactions and the rates of various activities of the organism could be expected to be proportional to the temperatures to which the animal is exposed. It is, however, well documented that a great variety of poikilothermic animals can compensate for temperature in various physiological activities (Bullock, 1955; Precht, Christophersen and Hensel, 1955; Prosser, 1964). Northern populations of the same or closely related species often have higher rates of activity than southern populations measured at the same temperature. As a result, northern and southern populations may have similar rates of activity although they are living in very different thermal environments (Bullock, 1955; Precht, 1958; Vernberg, 1962).

The compensation for temperature in physiological activities which may be encountered among populations from different latitudes is frequently found to be stable and the differences are assumed to be genetically determined. On the other hand, the response of an individual organism to temperature may be dependent upon the temperatures to which the animal was previously exposed. Any such acclimation effect must be ruled out before differences between geographically separated populations can be considered to have a genetic basis.

Several investigators have compared the metabolic rates of latitudinally or altitudinally separated populations of the same or different species of Anura. Thus, Tashian and Ray (1957) reported differences in the rates of oxygen consumption between adults of northern and southern species of frogs. In addition, Tashian and Ray (1957) compared northern and southern subspecies of *Bufo boreas* while Packard (1971) compared montane and piedmont populations of *Pseudacris triseriata*. Significant differences were not found in either case. However, Jameson, Taylor and Mountjoy (1970) report a great deal of variability in metabolic rates between populations of the frog, *Hyla regilla,* from localities extending from British Columbia to Baja California. They found no clear-cut correlation between latitude and metabolic rate but did find that frogs from localities which were similar with respect to climate showed similar degrees of metabolic adjustment and tended to differ markedly from frogs from climatically different regions.

In the examples cited, the possibilities that genetically fixed latitudinal and short-term acclimation effects would be confounded were reduced by comparing animals acclimated at the same temperature under controlled conditions in the laboratory. Nonseasonal thermal acclimation of the metabolic rate has been reported for a number of anurans and is often quite pronounced. A partial compensation for temperature in the metabolic rate has been reported for several species of *Rana* (Stangenberg, 1955; Rieck, Belli and Blaskovics, 1960; Jankowsky, 1960) and for *Bufo boreas* (Bishop and Gordon, 1957). In these anurans, animals which were acclimated at low temperatures were reported to have a higher metabolic rate when measured at an intermediate temperature than those acclimated at a higher temperature. On the other hand, *Rana esculenta* sampled in the summer (Locker and Weish, 1966; Stangenberg, 1955: Table 1) and *Acris crepitans* (Dunlap, 1969, 1971), have been reported to exhibit inverse compensation. In this case, frogs acclimated at high temperatures have higher metabolic rates when measured at an intermediate temperature than those acclimated at lower temperatures.

Considering the taxonomic diversity of the Anura and the wide-spread distribution of the order, there is a paucity of information on the relationship between latitude, climate and metabolic rates within the taxon. Furthermore, the majority of cases in which latitudinally related effects on metabolic rates have been reported have involved animals which exhibit partial compensation (Vernberg, 1962). There is, then, a need for more data on which to base hypotheses concerning the general significance of inverse compensation to those organisms in which it has been reported. Consequently, a comparison was made of the acutely measured metabolic rate–temperature curves of acclimated cricket frogs from two latitudinally widely separated populations. In this fashion it was expected that any obvious differences in acclimation pattern or in the metabolic responses of the two populations of frogs to changing temperatures would be demonstrated.

MATERIALS AND METHODS

Samples of cricket frogs were collected in July from near Vermillion, South Dakota (Latitude 42° 48′N, elevation 1,220 ft) and Austin, Texas (Latitude 30° 18′N, elevation 615 ft). Vermillion lies approximately 870 miles north of Austin.

Frogs from South Dakota were collected one to two days prior to placing them in the acclimation chambers. Texas frogs were collected, shipped to Vermillion via air express and placed in the acclimation chambers upon arrival. Groups of frogs were acclimated in the dark for 5–7 days (Dunlap, 1969) at experimental temperatures of 15° and 25° ± 1° C. They were maintained in loosely covered glass jars and had access to free water but were not fed during the course of acclimation. Oxygen consumption was measured for individual frogs using a refrigerated Gilson differential microrespirometer equipped with 100 ml flasks. Each flask received 5 ml deionized water in the animal chamber and carbon dioxide was absorbed by 1.5 ml 20% KOH placed in the side arm. The flasks were equilibrated for 30 min and readings were taken every 15 min for two hours. Stability of the system was routinely monitored by the insertion of a blank specimen vessel.

Metabolic rates are given as μl/g per hr STP of oxygen and are based on the average hourly uptake over the two hour period. These can best be considered as routine rates in the sense of Fry (1957) inasmuch as there was no control of spontaneous locomotor activity during the two hour period of determination. Measurements of oxygen consumption which are made within a few hours of transfer of the frogs from the acclimation temperature to the temperature of determination referred to as acute measurements and the corresponding rates as acute rates following the terminology of Bullock (1955). Acclimated rates refer to rates calculated for animals in which the acclimation temperature and the temperature of determination are the same.

In the determination of the acutely measured metabolic rate-temperature (R-T) curves, oxygen consumption was measured at one of seven temperatures (5, 10, 15, 20, 25, 30 or 35 ± 0.1° C) for frogs acclimated at 15 or 25 ± 1° C. Each combination of acclimation temperature and determination temperature for each of the two localities was represented by a sample of six frogs. A different group of frogs was used for each set of determinations and each frog was used only once. These data, then, are based on 84 different frogs from each of the two localities or a total of 168 frogs.

Animals collected from each locality were assigned to each of the individual groups on a random basis with the restrictions that there should be approximately equal proportions of males and females and equal proportions of three arbitrarily assigned size classes within each group. Frogs from South Dakota averaged larger (1.5 g body wt) than those from Texas (0.5 g). The statistical techniques used in the analyses are from Li (1957) and Ostle (1963).

TABLE I

Mean metabolic rates in μl/g per hr and their standard errors for Acris crepitans *from South Dakota and Texas. The frogs were acclimated at 15 and 25°C and determined at the temperatures indicated. Each mean is based on a sample of six frogs. The data have not been corrected for differences in body weight*

Locality	Acclimation temp.	Determination temperature						
		5	10	15	20	25	30	35
South Dakota	15	35 ± 1.0	67 ± 8.1	77 ± 5.9	108 ± 7.8	160 ± 8.4	236 ± 12.4	349 ± 15.9
	25	56 ± 3.7	102 ± 7.2	154 ± 14.8	169 ± 10.3	169 ± 9.6	249 ± 16.8	332 ± 11.9
Texas	15	33 ± 2.1	66 ± 5.6	67 ± 6.6	156 ± 13.0	174 ± 15.2	226 ± 25.6	374 ± 37.0
	25	44 ± 5.0	80 ± 8.7	110 ± 6.6	173 ± 16.5	234 ± 46.0	220 ± 15.1	296 ± 11.8

RESULTS

The mean metabolic rates of the Texas and South Dakota frogs are shown in Table I. Since the mean body weights of frogs from the two localities were markedly different and since metabolic rate may be a weight-dependent variable in cricket frogs (Dunlap, 1969, 1971), comparisons among sets of data for any one determination temperature were made using a 2 × 2 analysis of covariance. In each analysis, mean metabolic rates are compared for samples of frogs from each locality, acclimated at 15 and 25° C and determined at one of the seven temperatures. Prior to analysis, the data were subjected to a $\log_{10}$ transformation for both metabolic rate and body weight. This transformation has the double effect of reducing the heterogeneity of the variances and of transforming the regression lines to a more linear form (Dunlap, 1971). Also, prior to the analysis, the hypothesis that the regression coefficients of the regression lines being compared are equal was tested and accepted ($P > 0.1$) in all cases). The results of the analyses are shown in

Table II. Interaction was significant ($P < 0.05$) at determination temperatures of 20 and 35° C so these sets of data were analyzed further.

Locality comparisons

Differences in mean metabolic rates between Texas and South Dakota frogs were not significant at a determination temperature of 5° C or 25° C ($P > 0.25$ in each case). At 10 and 15° C, overall significance for locality was borderline ($P > 0.05$, < 0.1). If, however, metabolic rates of frogs acclimated at 25° C are compared separately from those of the 15° C frogs, the mean rate of the South Dakota frogs is significantly higher ($P < 0.05$) than that of the Texas animals. There is no significant difference attributable to locality when both samples are acclimated at 15° C and determined at either 10 or 15° C ($P > 0.25$). At 20° C only the frogs acclimated at 15° C exhibited a significant difference attributable to locality ($P < 0.005$) with the Texas frogs having the higher rate. For frogs acclimated at 25° C and determined at 20° C there is no significant difference attributable to locality ($P > 0.25$). At 30° C the metabolic rates of frogs acclimated at both temperatures were significantly different for locality, with the South Dakota frogs having the higher rates ($P < 0.025$). At 35° C there is no significant difference for the 15° C frogs ($P > 0.1$) but locality differences are significant for the 25° C frogs ($P < 0.025$). The South Dakota animals have the higher rate.

TABLE II

Mean squares, calculated F-ratios and their associated probabilities resulting from the 2 × 2 analysis of covariance of metabolic rates in Acris crepitans *following a log transformation of the data. The localities compared are Austin, Texas and Vermillion, South Dakota and the acclimation temperatures; 15 and 25°C. In each case metabolic rates were determined at the temperature indicated. For each analysis, the degrees of freedom are 1 and 19. Values in parentheses below the F-values represent probabilities*

Source of variation	Temperature of determination (°C)						
	5	10	15	20	25	30	35
Locality M.S.	0.0021	0.0387	0.0313	0.0069	0.0005	0.0466	0.0253
Acclimation M.S.	0.1890	0.0993	0.3462	0.0809	0.0148	0.0010	0.0242
Interaction M.S.	0.0005	0.0204	0.0135	0.0348	0.0054	0.0008	0.0128
Error M.S.	0.0111	0.0091	0.0100	0.0074	0.0145	0.0048	0.0025
F-ratios Locality	0.184 (>0.25)	4.251 (>0.05; <0.1)	3.127 (>0.05; <0.10)	0.908 (>0.25)	0.037 (>0.25)	9.765 (<0.01)	10.138 (<0.005)
Acclimation	16.950 (<0.001)	10.899 (<0.005)	34.629 (<0.001)	10.993 (<0.005)	1.023 (>0.25)	0.210 (>0.25)	9.722 (<0.01)
Interaction	0.048 (>0.25)	2.234 (>0.10)	1.350 (>0.25)	4.733 (<0.05)	0.374 (>0.25)	0.168 (>0.25)	5.153 (<0.025)

Acclimation comparisons

The metabolic rates of frogs acclimated at 15 and 25° C are significantly different at 5, 10 and 15° C ($P < 0.005$), with the frogs acclimated at 25° C having

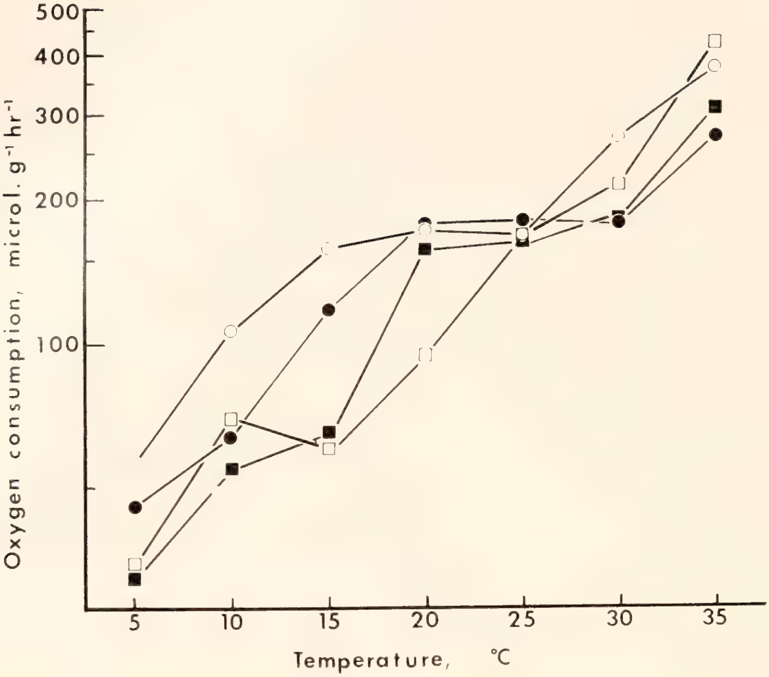

FIGURE 1. Routine oxygen consumption in *Acris crepitans* from Texas and South Dakota at various temperatures following acclimation at 15° and 25° C. Each point on the graph represents the predicted mean metabolic rate for frogs weighing 1 gram. Symbols representing the treatments are: Texas, 15° C (■); Texas, 25° C (●); S. D., 15° C (□); S. D., 25° C (○).

the higher rates. At 20° C acclimation effects are significant only for the South Dakota frogs ($P < 0.005$), with the 25° C acclimated frogs having the higher rates. At 25 and 30° C acclimation effects were not significant ($P > 0.25$) for either locality. At 35° C the South Dakota frogs showed no acclimation effects ($P > 0.1$) but the Texas animals did ($P < 0.025$). The frogs acclimated at 15° C have the higher rate.

The R-T curves for the four series (Texas frogs acclimated at 15 and 25° C, South Dakota frogs acclimated at 15 and 25° C) are shown in Figure 1. The mean rates used in the construction of the graph are the metabolic rates for frogs weighing 1 g predicted from the regression equation calculated from the data of each treatment. This figure illustrates the strikingly different metabolic responses of frogs acclimated at 15 and 25° C when oxygen consumption is measured at temperatures of less than 20° C for the Texas frogs and less than 25° C for the South Dakota frogs. Warm acclimated frogs from both Texas and South Dakota have a higher metabolic rate in this range of temperatures than do cool acclimated animals. The magnitude of the acclimation response is, however, greater for the South Dakota than for the Texas frogs. This is shown especially well when the R-T curves for the frogs acclimated at 25° C are compared. For frogs acclimated at 25° C, South

Dakota frogs have consistently higher metabolic rates than do Texas frogs when metabolic rates are determined at 5, 10, and 15° C. At determination temperatures above 25° C there are clearcut differences in metabolic responses of frogs from the two localities, with the Texas animals having the lower rates. At determination temperatures of 30 and 35° C no acclimation effects can be demonstrated for the South Dakota frogs. At 35° C, however, the cool acclimated Texas frogs have a slightly higher metabolic rate than the warm acclimated frogs.

Effects of acclimation on sensitivity of metabolic rates to temperature change

Since the curves of Figure 1 are plotted semilogrithmically, segments of lines with equal slopes represent temperature regions with equal Q_{10} values. Q_{10} values were calculated at 5° C intervals using the equation

$$Q_{10} = \left(\frac{V_1}{V_2}\right)^{[10/(t_1-t_2)]}$$

where V_1 and V_2 are metabolic rates corresponding to the temperatures t_1 and t_2. The calculated Q_{10} values for South Dakota and Texas animals acclimated at 15 and 25° C are shown in Table III. Chemical reaction rates are usually more than doubled per 10° C increase in temperature (Prosser and Brown, 1961). Hence, a Q_{10} of less than 2 would indicate a relative insensitivity of metabolic rate to temperature change within the temperature range indicated. As might be surmised from an examination of the R-T curves, Q_{10} values of approximately 1.0 obtain between 15 and 25° C for the South Dakota frogs acclimated at 25° C and between 20 and 30° C for the 25° C Texas frogs. When frogs from both localities are acclimated at 15° C, Q_{10} values are low between 10 and 15° C. In the Texas animals the Q_{10} is low between 20 and 30° C as well. The latter plateau on the 15° C R-T curve is barely noticeable for the data from South Dakota frogs. For frogs acclimated at 15° C, segments of the R-T curves characterized by very low Q_{10} values are often immediately preceded by segments with high values, *e.g.*, the curve for Texas frogs between 20–30° C. In the R-T curves for frogs acclimated at 25° C, on the other hand, the transition between segments with high and those with low

TABLE III

Q_{10} values determined at 5°C intervals for Acris *from South Dakota and Texas acclimated at 15 and 25°C*

Acclimation temperature (°C)	Temperature interval (°C)					
	5–10	10–15	15–20	20–25	25–30	30–35
South Dakota frogs						
15	4.109	0.729	2.512	3.066	1.621	3.857
25	3.478	2.226	1.171	0.956	2.582	1.932
Texas frogs						
15	2.759	1.395	5.774	1.107	1.241	2.782
25	2.019	3.349	2.256	1.010	1.006	2.226

Q_{10} values is less abrupt. The acclimated R-T curves for South Dakota and Texas *Acris* between 15 and 25° C are essentially identical and have a Q_{10} value of 2.8.

Discussion and Conclusions

Although the forms of the acutely measured R-T curves are complex, the curves of the South Dakota and Texas frogs in July show many similarities. They are also similar in form to those of South Dakota frogs determined in late May and early June (Dunlap, 1971). In each case, the same basic acclimation pattern is found. In Precht's (1958) terminology, the metabolic rates of *Acris* exhibit type V (inverse) compensation when the rates of warm acclimated (25° C) frogs are compared to those of cold acclimated (15° C) frogs at the lower temperature. Type IV (no) compensation, however, is evident when the metabolic rates of cold acclimated frogs are compared to those of warm acclimated animals at the higher temperature. This pattern is reflected in the intersection of the R-T curves which occurs at 20° C for the Texas frogs and 25° C for the South Dakota frogs. At temperatures below the intersection, warm acclimated frogs have a higher rate of oxygen uptake than do the cold acclimated animals. For South Dakota frogs, no acclimation effects are evident at the intersection of the R-T curves or at higher temperatures. Acclimation effects are absent between 20 and 30° C for Texas frogs. At 35° C, however, the cold acclimated Texas frogs have slightly higher rates than the warm acclimated frogs. As a result of this complex pattern of acclimation, the metabolic rates of warm acclimated frogs remain stable and Q_{10} values approximate 1 over a range in body temperature of at least 10° C. See Dunlap (1971) for a more detailed analysis of this acclimation pattern.

Cricket frogs are active both night and day and during the day may frequently be encountered basking in the sun at margins of ponds and small streams. Fitch (1956) reported that in northeastern Kansas, of 102 body temperatures recorded for *Acris* throughout the year, over half were between 28.0 and 31.7° C. In cool weather he found that body temperatures often exceeded air temperature by 10° C or more, but in warm weather the frogs basked less and body temperatures were usually nearer air temperature. Brattstrom (1963), on the basis of his own and Fitch's data, suggested that *Acris crepitans* may engage in thermoregulatory movements involving alternate basking and immersion in the water during the day and repeated immersion at intervals during the evening while water temperatures are higher than ambient air temperatures. The body temperatures of the frogs fluctuate less both daily and seasonally than might be expected on the basis of ambient temperature alone. Thus, Brattstrom (1963) measured body temperatures of Texas *Acris* between 0900 and 2300 hr and reported a mean body temperature of 24.9° C with a range from 30.0–22.0° C. During the same period, air temperature varied from a high of 27.0° C in the afternoon to a low of 21.5° C at night and water temperature varied from 27.2° C in the afternoon to 23.8° C at night. These data, then, suggest that *Acris* can, by behavioral thermoregulation, maintain a body temperature close to 30° C while the sun is shining. At night, however, body temperatures drop to levels that, on the average, lie between ambient air and water temperatures.

As we have seen, Texas frogs acclimated at 25° C are metabolically relatively insensitive to temperature change between 20 and 30° C. All of the values for body temperature given by Brattstrom and the bulk of Fitch's warm season values fall within these limits.

In an earlier paper (Dunlap, 1971), I suggested that inverse compensation together with the associated regions of metabolic insensitivity to temperature change enabled warm acclimated summer animals to maintain a high and relatively stable metabolic rate in spite of fluctuations in body temperature which occur between day and night and from day to day. Conversely, under conditions of constant and low temperature, as could be expected to occur during hibernation, the frogs would be cold acclimated and the relatively low rate of metabolism would result in a reduced rate of energy expenditure from energy reserves during the dormant period. The above interpretation is consistent with the available data on the body temperature of cricket frogs under field conditions. This model is based on the assumption that the frogs are warm acclimated throughout the summer. That is, they acclimate to their basking temperature or to a mean body temperature rather than to the lower temperature reached during the daily thermoperiod. Although I am aware of no experimental evidence for this with respect to metabolic acclimation, the work of Hutchison and Ferrance (1970) and Seibel (1970) lends credence to such an assumption. These investigators reported that in the frog, *Rana pipiens*, the critical thermal maximum responds to the maximum temperature when the frog is subjected to a daily thermoperiod.

In comparing the R-T curves of the 25° C acclimated frogs from South Dakota and Texas, it may be seen that the region of relative metabolic insensitivity to temperature extends from 20 to 30° C for the Texas animals and from 15 to 25° C for the South Dakota animals (Fig. 1). Furthermore, the metabolic rates of warm acclimated South Dakota frogs are higher than those of Texas frogs at all tested temperatures below 20 and above 25° C. These differences would seem to be accounted for by a simple translation of the R-T curve of the South Dakota frogs 5° to the left as compared to the Texas population. If the warm acclimated curve for the South Dakota frogs is moved 5° C to the right the two curves are essentially superimposed.

The mean maximum and minimum temperatures for July are 31.5 and 17.2° C, respectively, for Vermillion, South Dakota (Spuhler, Lytle and Moe, 1967) and 34.6 and 23.2° C for Austin, Texas (Blood, 1960). The differences noted above between the warm acclimated R-T curves of the northern (South Dakota) and the southern (Texas) populations, taken with the climatological data are consistent with the hypothesis of the role of metabolic patterns in the maintenance of metabolic stability in a varying thermal environment. If the thermoregulatory abilities of the two populations of frogs are similar, one could expect that in July the nocturnal body temperatures of the South Dakota frogs would, on the average, be lower than those of the Texas frogs. Yet, due to the shift of the curves for the South Dakota frogs to the left, nocturnal metabolic rates could be expected to average about the same for the two populations. Conversely, the mean maximum body temperatures of the Texas frogs might be expected to average somewhat higher during the day than those of the South Dakota animals. Under these conditions the depression of the metabolic rates of the Texas frogs at higher temperatures relative to the South

Dakota frogs would contribute toward the stabilization of the metabolic rates at higher body temperatures.

Except for the 20° C determination temperature, South Dakota and Texas frogs acclimated at 15° C do not differ significantly from each other. At 20° C the 15° C Texas animals behave metabolically as warm acclimated animals while the 15° C South Dakota frogs still have depressed rates. This difference in metabolic rates between samples from the two localities, if it proves to be characteristic of winter as well as summer animals, might be related to differences in degrees of winter activity of frogs at the two localities. In the Vermillion area *Acris* spends four to five months hibernating in ponds with body temperatures probably approximating 3° C (Dunlap, 1971). In the vicinity of Austin, frogs are active during all months of the year although the number of active frogs decreases in December and January (Pyburn, 1958). At temperatures above 5° C Dunlap (1971) found no significant differences in the metabolic rates of frogs acclimated at 5 and 15° C. If the acclimation pattern remains constant throughout the year, the abrupt increase in metabolic rate between 15 and 20° C in the Texas frogs would allow cold acclimated winter frogs to attain high metabolic rates and presumably high activity rates on warm sunny winter days. On the other hand, the South Dakota frogs would retain the depressed metabolic rates even in the face of brief periods of warm weather. If the northern populations were active during the occasional warm winter periods when the ice melts they would still be faced with a scarcity of food and the danger of being excluded from the hibernation pools when freezeup occurs again. Consequently, the greater constraints placed on the attainment of a high metabolic rate in the northern as compared to the southern population at moderate temperatures is, it seems to me, consistent with an adaptive interpretation of the metabolic and acclimation pattern.

The acclimated R-T curves between 15 and 25° C are almost identical for the two populations and with a Q_{10} of 2.8 exhibit no metabolic compensation for temperature. Dunlap (1971) has suggested that this may relate to the fact that these small frogs live in an environment of pronounced thermal instability. Consequently, body temperature could be expected to equal acclimation temperature for only a part of the time that the frog is active. This is, of course, quite different from Bullock's (1955) model which applies to environments such as the oceans in which environmental temperatures are much more stable and change slowly with the seasons. Under these conditions Bullock suggested that the acclimated R-T curves would exhibit temperature compensation.

The Texas and South Dakota populations of *Acris crepitans* are both placed in the subspecies *A. c. blanchardi* (Conant, 1958). This arrangement is supported on the basis of their general morphological (Harper, 1947) and biochemical (Dessauer and Nevo, 1969) similarity. However, populations of frogs from Texas tend to be more polymorphic for transferrins and slow esterases than the South Dakota populations (Dessauer and Nevo, 1969). Further, the two populations differ with respect to the predominance of one or the other of the alleles of the H subunit locus of lactate dehydrogenase (Salthe and Nevo, 1969). The one South Dakota population studied is monomorphic for one form of HLDH while Texas populations tend to be either monomorphic for the other form or are polymorphic. This is of special interest in view of the growing body of evidence for the function of enzyme variants in minimizing the sensitivity of a given reaction to temperature. Hochachka and Somero (1968), for example, suggest that enzyme variants (*e.g.*, LDH enzymes) are induced during short-term acclimation and the same variants may be differentially selected by different populations during evolutionary adaption to different regional climates.

The results of the present study are in general agreement with the studies cited above. The South Dakota and Texas frogs show an overall similarity in acclimation pattern, as reflected in the acutely measured R-T curves and in terms of their metabolic rates within the range of temperatures encountered in the course of their activities. The differences described here are suggestive of selection acting as fine tuning mechanism, adjusting the metabolic rate-temperature relations of the different populations to their specific environments.

I wish to thank Mr. Joe Ideker for supplying the Texas frogs used in this study. This investigation was initiated under Grant No. GB-5298 from the National Science Foundation.

SUMMARY

Samples of cricket frogs were collected in Texas and in South Dakota in July and acclimated for 5–7 days at 15 and 25° C. Routine metabolic rates were determined at 5, 10, 15, 20, 25, 30, and 35° C for samples from both localities acclimated at both temperatures. Sets of data from samples determined at each of the seven determination temperatures were subjected to a 2 × 2 analysis of covariance.

For both localities, warm acclimated (25° C) frogs had higher metabolic rates than cool acclimated (15° C) frogs at determination temperatures below 20° C (Texas) or below 25° C (South Daokta). At determination temperatures from 25 to 35° C (South Dakota) and 20 to 30° C (Texas), acclimation effects were not significant. At 35° C, Texas frogs acclimated at 15° C had a higher metabolic rate than those acclimated at 25° C.

Frogs acclimated at 15° C showed no significant locality effects when determined at 5, 10, 15, 25, and 35° C. At 20° C, the Texas frogs acclimated at 15° C had a significantly higher rate than the corresponding South Dakota frogs, while at 30° C, the South Dakota frogs had the higher rate. When both were acclimated at 25° C, the South Dakota frogs had a significantly higher rate than those from Texas at determination temperatures of 10, 15, 30, and 35° C. At 5, 20, and 25° C, however, no significant locality effects were apparent.

On the acutely measured R-T curves of cricket frogs acclimated at 25° C, there is a region of pronounced metabolic insensitivity to temperature in which region the Q_{10} approximates 1.0. The region lies between determination temperatures of 15–25° C for the South Dakota frogs and between 20–30° C for the Texas animals. Frogs from both localities acclimated at 15° C have a low Q_{10} value between 10–15° C and the Texas frogs have another one between 20 and 30° C. The differences noted above between the warm acclimated R-T curves of the northern and southern populations, taken in conjunction with published data on body temperature-environmental temperature relationships in *Acris* and with published climatological data, are consistent with the hypothesis of the role of metabolic patterns in the maintenance of metabolic stability in a varying thermal environment.

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HYPERBARIC OXYGEN AND EMBRYONIC DEVELOPMENT IN *ARBACIA PUNCTULATA*[1]

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Oxygen toxicity to living systems has been recognized since the classical studies of Bert (1878), but relatively little attention has been directed toward determining the effects of oxygen at high pressure upon developing systems. Such studies do include those of New and Coppola, 1970 (rat); Pizzarello and Shircliffe, 1967 (chick); Ferm, 1964 (hamster); Rosenbaum, 1960, Malamed, 1957, Nelson, 1949, and Rauber, 1884 (frog); and Rosenbaum and Wittner, 1960 (sand dollar). Miller, Miller, DeSha and Heidger (1969) investigated the effects of hyperbaric oxygen (HBO) upon embryonic development in the hydroid, *Tubularia,* and demonstrated that differentiation is blocked by exposure to pure oxygen at pressures of 2 to 4 atmospheres absolute (AA). Blockage of differentiation was accompanied by inhibition of succinic dehydrogenase activity. In view of the high succinic dehydrogenase activity which prevails during portions of sea urchin development (Gustafson and Hasselberg, 1951), it was of interest to extend our investigation to another phylum, the Echinodermata, and to a species from which large numbers of embryos are readily obtained and are routinely reared *in vitro.* This paper presents the results of studies of the effects of hyperbaric oxygen upon the embryonic development of *Arbacia* from fertilization to the time of formation of the pluteus larva.

MATERIALS AND METHODS

Specimens of *Arbacia punctulata* collected by the Supply Department of the Marine Biological Laboratory were used in all experiments. Gametes were shed by electrical stimulation (Harvey, 1956), and the eggs, pooled from several females, were suspended in filtered sea water at room temperature and were washed several times. They were inseminated with a dilute sperm suspension. The percentage fertilization was assessed 10 minutes post-insemination by observation of the elevation of the fertilization membrane, and all samples showing less than 95% fertilization were discarded.

The fertilized eggs were then distributed to finger bowls or Petri dishes such that a single layer of zygotes was distributed over the bottom of the container. The zygotes then were transferred to a 16° C constant temperature room, and were assigned randomly to control or experimental groups. Control groups were allowed to develop in air at 16° C, and experimental groups were subjected at the same temperature to 100% oxygen at 3 AA, within a Bethlehem hyperbaric chamber. Compression and decompression of the chamber were performed slowly such that no temperature changes occurred. The chamber was thoroughly flushed with gas before each compression. All times reported in this study were recorded from the time of insemination, taken to correspond closely with the time of fertilization.

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[1] Supported by NIH Grant 5T1 GM 00793 to J. A. Miller and a Faculty Research Award from the University of Maine to R. G. Summers.

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Sampling

Sampling was accomplished by two means. In the case of a continuing culture of embryos, an aliquot of embryos and sea water was withdrawn by pipette after the entire culture had been agitated by a stream of air. In the case of terminal samples, the entire culture was fixed by the addition of several ml of 5% glutaraldehyde in sea water after a sample had been removed for assessment of viability. Two investigators routinely checked the samples by the double blind technique. Staging of embryos was carried out according to Harvey, 1956.

Histological studies

Control and experimental embryos were fixed by immersion in 3% glutaraldehyde in sea water for 24 hours. The embryos were washed in filtered sea water, dehydrated in alcohols, cleared in xylene, and embedded in Tissuemat (Fisher). Seven-micron sections were cut and were stained with Ehrlich hematoxylin and alcoholic eosin-Y. Both embryos and histologic preparations were examined and photographed using a Reichert Zetopan photomicroscope.

Three principal series of experiments were performed: *Series 1*, To determine the effect upon developmnt of constant exposure to hyperbaric oxygen. Embryos were exposed to 3 atmospheres absolute oxygen for 72 hours, post-fertilization age. Controls developed in air at normobaric pressure. *Series 2*, To determine the reversibility of effects found in Series 1 experiments. Embryos were exposed to 3 atmospheres absolute oxygen for 12, 24, 36 or 48 hours. They were removed from the hyperbaric chamber and were then allowed to develop in air for up to 144 hours post-fertilization age. Controls developed in air at normobaric pressure. *Series 3*, To determine the time during development at which hyperbaric oxygen first exerts its effect. Embryos were allowed to develop in air for 12, 18, 24, or 36 hours and were then subjected to hyperbaric oxygen until 72 hours, post-fertilization age. Controls developed in air at normobaric pressure.

Special controls

In addition to the controls outlined above, two series of controls were performed for the following reasons.

Control series 1. To exclude the possibility that observed results might be caused by exposure to 100% oxygen (Linde), and not by oxygen under pressure, embryos were incubated under 1 AA pure oxygen for 72 hours.

Control series 2. To exclude the possibility that observed results might be caused by increased pressure, and not to oxygen under pressure, embryos were incubated in the hyperbaric chamber under 1 AA pure oxygen, and 2 AA pure nitrogen for 72 hours.

RESULTS

The key developmental stages and the time at which each was observed during development at 16° C are as follows: early blastula, 6 hours; hatching blastula, 12 hours; mesenchyme blastula, 24 hours; gastrula, 30 hours; late gastrula-early prism, 36 hours; and young plutei, 48 hours (see Figs. 9, 10 and 11).

Series 1 experiments

The results of this series are summarized in Table I. Both control and hyperbaric groups developed in synchrony to the gastrula stage of development. At the time of late gastrula, however, the hyperbaric embryos appeared arrested and did not proceed to form prisms or plutei. Rather, by 48 hours, most of the embryos had lost the archenteron structure distinguishing the gastrula, and were, as assessed by optical section of whole embryos, "regressing" to a configuration reminiscent of the blastula stage, but with a loose, disorganized population of cells within the central lumen. This impression which was gained from the examination of whole embryos (see Figs. 4 and 5), was verified by histological preparations (see Figs. 7 and 8). These figures illustrate that the relationships of the peripheral cells of the embryo appear to have been maintained, but that the archenteron structure was disorganized and was not identifiable as such. It is noteworthy that in each of the samples studied in this series both control and hyperbaric groups contained comparable numbers of dead or unclassifiable embryos. Therefore, HBO did not exert an immediate lethal effect, but produced a specific alteration in morphogenetic pattern. However, if the embryos were maintained in HBO continuously for 72 hours, the embryos disaggregated and died.

TABLE I

The effect upon Arbacia *development of exposure to 3 AA oxygen for various lengths of time*

Length of exposure to 3 AA O_2	Treatment	Per cent of sample*						
		Dead or abnormal	Unhatched blastula	Mesenchyme blastula	Normal gastrula	Abnormal gastrula or regressing gastrula	Prism	Pluteus
12 hr	Control	6	94					
	Hyperbaric	5	95					
24 hr	Control	6	0	94				
	Hyperbaric	7	0	93				
36 hr	Control	6	0	0	94			
	Hyperbaric	6	0	0	80	14		
48 hr	Control	4	0	0	2	0	7	87
	Hyperbaric	5	0	0	1	94	0	0

* Minimum of 500 embryos counted in each sample.

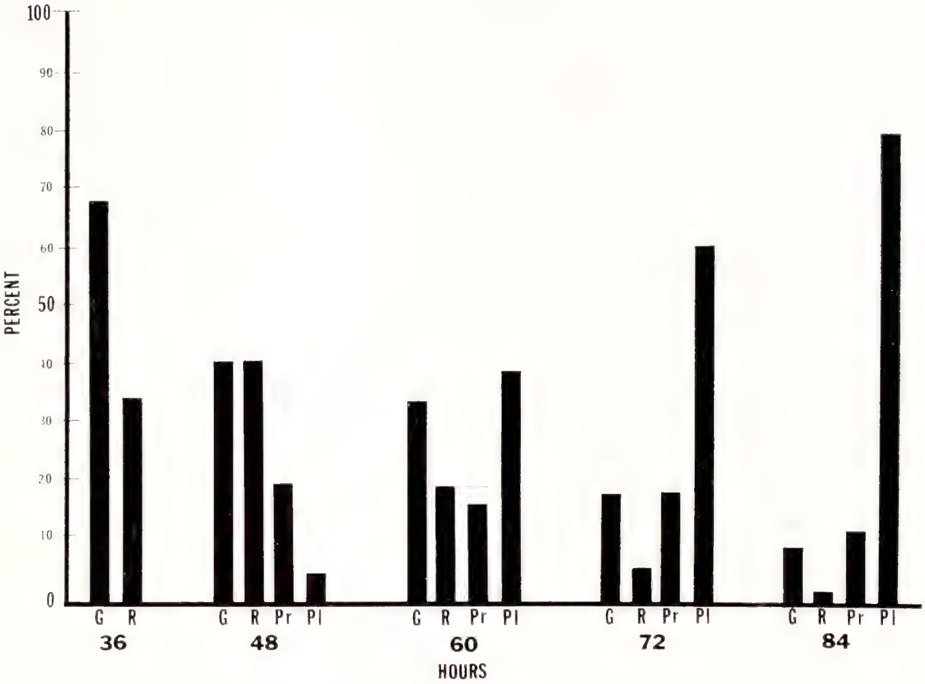


FIGURE 1. Bar graphs showing the per cent of embryos at given developmental stages at various times following removal of the embryos from 3 AA oxygen at 36 hours post-fertilization age. Abbreviations are: G = gastrula; R—regressing gastrula; Pr—prism stage; Pl—pluteus. A minimum of 200 embryos was counted at each age.

Series 2 experiments

Embryos were exposed to HBO for 12, 24, 36 or 48 hours and were then removed to air and subsequent development was observed.

(a) *Embryos removed at 12 or 24 hours.* Embryos exposed to HBO during the first 12 hours of development only were not altered morphologically, and developed at the same rate as did controls. Development was retarded in embryos exposed to HBO for the first 24 hours of development only, retardation being evidenced between the time of removal (mesenchyme blastula stage) and pluteus formation. No abnormal morphological alterations were detected, however. By 60 hours, nearly all such embryos had reached young pluteus stage. This represents a delay of 12 hours beyond the time at which all control embryos reached this stage (48 hr).

(b) *Embryos removed at 36 hours.* (See Fig. 1) At the time of removal, the embryo population consisted almost entirely of morphologically normal gastrulae, plus blastula-like forms designated "regressing gastrulae." No prism stages were seen; by 48 hours following removal, 17% of the population consisted of prisms, and 3% of normal young plutei. The relative numbers of prism and pluteus stages which were seen increased through 84 hours, when the experiment was terminated with over 90% of the embryos beyond gastrulation stage, *i.e.*, in prism or pluteus stages. The per cent of dead or unclassifiable embryos at all times studied was never more than 6% of the total population, and is not separately plotted in the text figures.

(c) *Embryos removed at 48 hours.* (See Fig. 2) At the time of removal from HBO, 37% of the embryo population was classified as regressing gastrula. A large proportion of the embryos classified as gastrulae were not normal gastrulae, but showed varying degrees of internal disorganization. For purposes of uniformity of classification, however, any embryo showing any evidence whatsoever of archenteron structure was placed in the gastrula category. Twenty-four hours following removal, a small population (2%) of prism stages was seen. Between 72 and 96 hours, plutei were formed, and the prism population increased to 11%. The appearance of the later stages of development was accompanied by a decrease in the regressing gastrula population from 41% at 72 hours to 17% at 96 hours. Gastrulae at this time appeared normal. The decline in gastrulae and regressing gastrulae and the increase in numbers of later embryonic stages continued, until at 144 hours, 94% of the population had progressed past the gastrula stage into prisms or plutei.

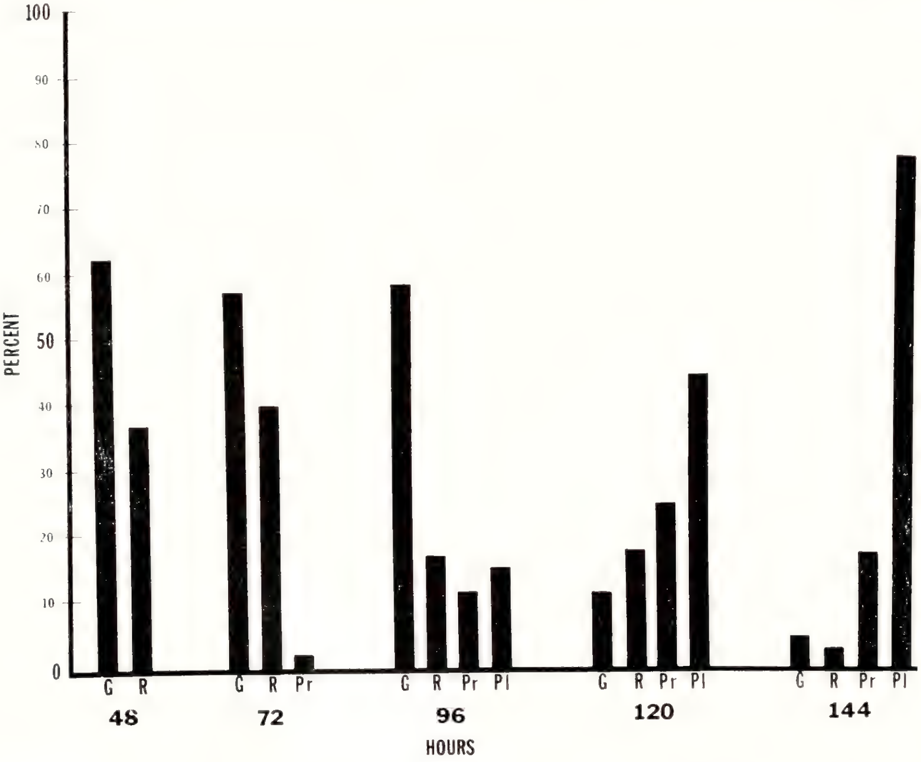


FIGURE 2. Bar graphs showing the per cent of embryos at given developmental stages at various times following removal of the embryos from 3 AA oxygen at 48 hours post-fertilization age. Abbreviations as in Figure 1. A minimum of 200 embryos was counted at each age.

Series 3 experiments

Table II summarizes the per cent of embryos reaching developmental stages 72 hours post-fertilization when exposed to HBO at various times in development. Embryos exposed following 12 hours of development in air (hatching blastula stage) gastrulated at the same time as did controls, but formed the blastula-like structures termed "regressing gastrulae" and showed early evidence of disaggregating by 72 hours. Unlike the regressing gastrulae seen in Series 1, rudimentary spicules were occasionally observed in this group embedded within the structure of the regressing gastrulae. Embryos exposed to HBO at 18 hours (having reached the swimming blastula stage), or at 24 hours (having reached mesenchyme blastula stage) were delayed in development, but showed a low percentage of regressing gastrulae, and a high percentage of normal gastrulae, prisms, and plutei at 72 hours. Embryos exposed following 36 hours of development in air (having reached the gastrula stage) progressed uninhibited to form young plutei by 48 hours. The plutei from this group when observed appeared morphologically normal but swam very slowly in comparison with controls. Further, normal, young plutei treated with HBO at 48 hours post-fertilization age were observed at 72 hours to swim much more slowly than did controls. These latter observations may possibly reflect a general depression of metabolic activity caused by HBO.

TABLE II

The effect upon Arbacia *development of exposure to 3 AA oxygen at various post-fertilization ages*

Age when first exposed to 3 AA oxygen	Per cent of embryos in stages at 72 hours of age*				
	Dead	gastrula	Regressing gastrula	Prism	Pluteus
12 hours	5	5	90	0	0
18 hours	1	24	9	65	1
24 hours	2	22	7	59	10
36 hours	3	0	0	2	95

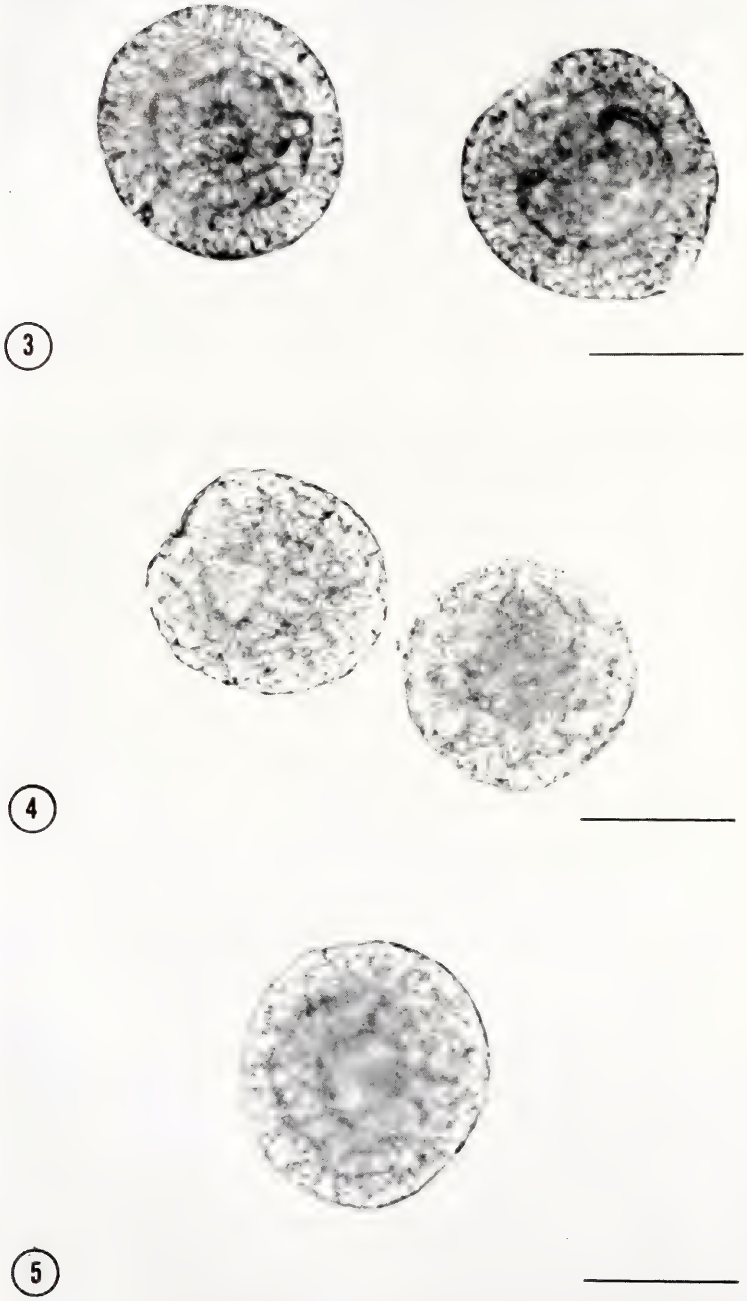
* A minimum of 200 embryos was counted for each age.

Controls

Embryos reared in 1 AA pure oxygen or in 1 AA pure oxygen plus 2 AA nitrogen did not differ in morphology or in developmental rate from embryos reared in air at normobaric pressure (see Figs. 11 and 12).

DISCUSSION

The patterns of effects on development of HBO are unique in the two species which we have studied. In contrast with toxic agents which produce death, differential inhibition, differential tolerance, conditioning or recovery depending upon the concentration and duration of the exposure (Child, 1941), hyperbaroxia appears



Figures 3-5.

to block development totally for extended periods of time and yet permits total or nearly total reversibility in its effects. The reversibility in *Tubularia* development is striking with total blockage of differentiation for a period of as much as five days followed by the formation of completely normal actinula larvae (Miller *et al.* 1969). In *Arbacia,* exposures which arrested gastrulation and induced apparent dedifferentiation and regression of the developing mesoderm and entoderm never-the-less were followed by complete recovery of normal morphology in more than 90% of the embryos. There were virtually no inhibited larvae with reduced oral lobes such as are characteristic of embryos exposed to KCN, $CuSO_4$, LiCl and many other toxic agents (*cf.* Fig. 47, page 199, Child, 1941).

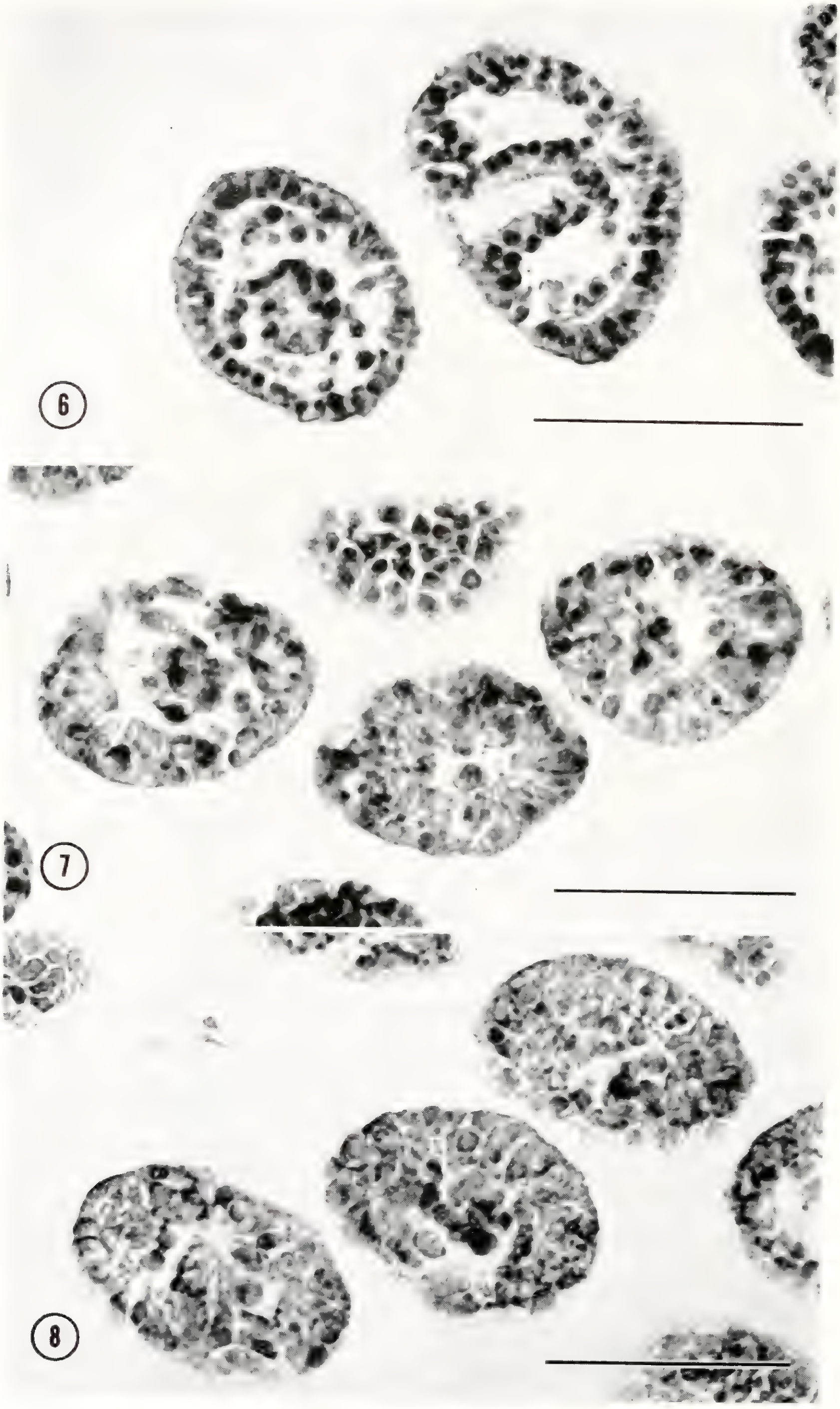
This suggests that the blockage which was produced by HBO did not induce the production of toxic substances which altered any future differentiation. Instead, it appears that, although blocked temporarily, their biochemical systems were not permanently injured, and could redifferentiate as soon as the excess oxygen was removed. These findings are consistent with the concept that the fundamental effect of hyperbaric oxygen is to oxidize enzymes which are active in the reduced state but are inactivated when in the oxidized condition (Haugaard, 1968).

Hyperbaric oxygen has been shown to inactivate sulfyhydryl-containing enzymes including succinic dehydrogenase (Haugaard, 1968; Davies and Davies, 1965). It is significant that this enzymatic activity has been shown to be high during gastrulation in the sea urchin (Gustafson and Hasselberg, 1951). A second enzymatic activity which is high in activity during gastrulation in the sea urchin is that of cathepsin II, which is low in activity up to the mesenchyme blastula stage, but which undergoes a sharp rise after this stage (Gustafson and Hasselberg, 1951). This enzyme is considered sulfhydryl-dependent (Fruton, Irving and Bergmann, 1941) and is known to be inhibited by HBO (Davies and Davies, 1965; Rosenbaum, 1960). Furthermore, it has been demonstrated that the flavoprotein, diaphorase, is inactivated by oxidation (Williams, 1965). Diaphorase-dependent dehydrogenase activities, such as those of glucose-6-phosphate dehydrogenase and malic dehydrogenase, might thus be indirectly inhibited under HBO. In this regard, it is significant that glucose-6-phosphate dehydrogenase has been shown in *Paracentrotus* to be maximally active immediately preceding, and through the first half of gastrulation (Backstrom, 1959); malic dehydrogenase activity rises during the mesenchyme blastula stage, gastrulation and later stages, paralleling the rise in succinic dehydrogenase activity (Gustafson and Hasselburg, 1951). The blockage of such enzymatic activities during development would be expected to disrupt development during the stages at which they are highly active; this correlates well with our observations of blockage within gastrula stage of HBO-treated embryos. Direct biochemical analysis of enzymatic activity during the course of HBO treatment of sea urchin embryos is in progress.

FIGURE 3. Control gastrulae, 36 hours post-fertilization age. Embryos were reared in air at normobaric pressure at 16° C; whole mount. Scale on all photomicrographs equals 50 μ.

FIGURE 4. Embryos reared in HBO at 3 AA, 16° C, for 36 hours: "regressing gastrula" stage. Archenteron structure is indistinguishable in these embryos. Compare with control embryos shown in Figure 3; whole mount.

FIGURE 5. Embryo reared in HBO at 3 AA, 16° C, for 48 hours. Morphology is similar to the regressing gastrulae shown in Figure 4; no additional morphological changes are observed with the 12 hours of additional exposure to HBO; whole mount.



Figures 6–8.

Our studies correlate well with those of Rosenbaum (1960) who reported arrest in the gastrula stage in frog embryos treated with HBO, and with those of Malamed (1957) who observed similar blockage in *Rana pipiens* embryos following incubation of eggs in a medium through which oxygen was bubbled. No studies have been made of the protection afforded embryos from the effects of HBO by BAL, crysteine, or glutathione (Wittner and Rosenbaum, 1958a, 1958b).

Series 1

Even though the "regressing gastrula" embryos which were observed at both 36 and 48 hours were indistinguishable morphologically from each other (compare Figs. 4 and 5, and Figs, 7 and 8), the 48-hour embryos were much more severely inhibited, as evaluated in terms of the time necessary to complete development through the pluteus stage (see Figs. 1 and 2). The population of embryos incubated in HBO for 48 hours required approximately twice the time, following removal from the chamber, to complete development through the pluteus stage as did those which were exposed to HBO for 36 hours. From observations of whole mounts, it appeared that the internal rearrangement of cells during recovery of the regressed gastrulae might not be dissimilar to that observed by Giudice and Mutolo (1970) in sea urchin embryos reaggregating from dissociated cells. These authors have shown that the intestine which develops from reaggregated spheres of cells does not form by invagination, as in normal development, but by the internal rearrangement of cells about a space destined to become the intestinal lumen. However, a detailed histological study of the reversal of inhibition seen in the present study remains to be performed.

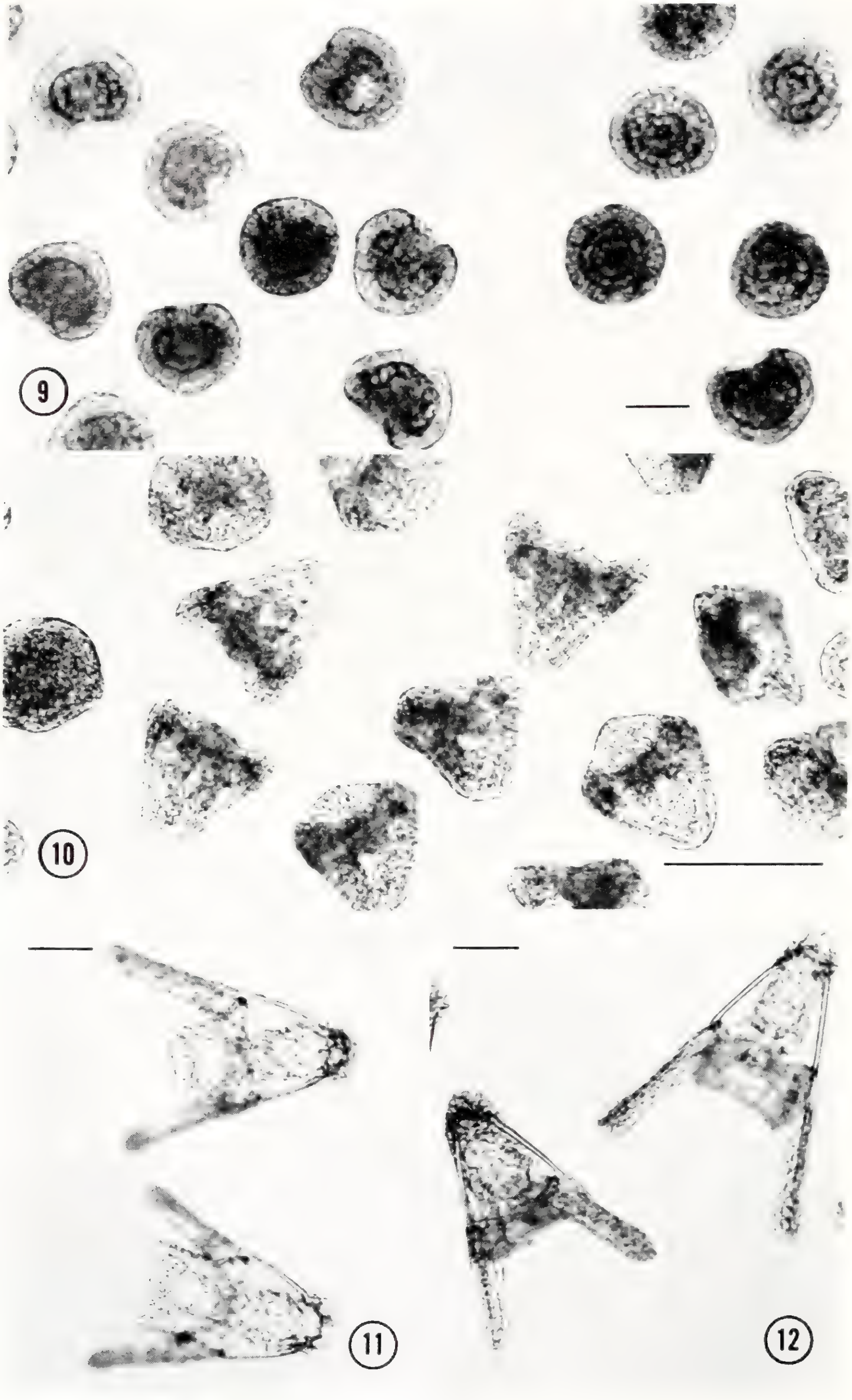
Series 2 and 3

It is instructive to examine the results of Series 2 and 3 in terms of assessing the time at which HBO may exert its inhibitory effect. Hyperbaric oxygen exerted no evident inhibitory effect upon embryos exposed continuously for 12 hours following fertilization; by 24 hours, however, an inhibition to development was manifested by the delay in the time of pluteus formation. The inhibition was observed to be more severe following 36 or 48 hours and was accompanied by the characteristic morphology of the "regressing gastrula." There would appear, therefore, to be no residual inhibition of processes regulating pluteus formation from only 12 hours of exposure to HBO. Alternatively, those processes essential to normal gastrulation and pluteus formation and susceptible to HBO inhibition may not be operative at such early stages. These processes may differentiate at

FIGURE 6. Histological section of control gastrulae reared in air at normobaric pressure for 36 hours at 16° C. Note the typically prominent archenteron structure and associated mesoderm; 7-micron section, hematoxylin and eosin stain.

FIGURE 7. Histological section of "regressing gastrulae." Fertilized eggs were exposed continuously to 3 AA oxygen for 36 hours at 16° C. Note the absence of an organized archenteron and the "blastula-like" appearance of the embryos; *cf.*, Figure 6; 7-micron section, hematoxylin and eosin stain.

FIGURE 8. Histological section of "regressing gastrulae." Fertilized eggs were exposed continuously to 3 AA oxygen for 48 hours at 16° C. Note morphology similar to that in Figure 7 above; 7-micron section, hematoxylin and eosin stain.



Figures 9–12.

later stages, and be inhibited by exposure to HBO later in development, in the mesenchyme blastula stage. It should be noted, however, that HBO may possibly exert a generalized depressive effect upon the metabolism of plutei; this is perhaps reflected in the slow swimming movements of morphologically normal plutei which were evident after prolonged exposure to HBO.

From Series 3 it may be inferred that embryos which have reached late gastrula (36 hr) prior to exposure to HBO have already been "programmed" for pluteus formation, and HBO at 3 AA exerts no effect upon this stage of morphogenesis. No information, however, is available in this system concerning possible delays in the action of HBO, as is seen in mammals (Haugaard, 1968). The possibility of such delays in effective inhibition must be investigated before a valid timetable of inhibition may be established.

Although no detailed comparative study of the initial cleavage patterns of control and HBO-treated embryos was made, no obvious effect of HBO upon cleavage pattern was noted in the present study, and both HBO-treated and control embryos developed synchronously until the time of gastrulation. These findings are in accord with those of Rosenbaum and Wittner (1960) who found in *Echinarachnius* that 4-hour exposures to oxygen at 3 to 4 atmospheres induced no anomalies of cleavage or development within the first 8 hours of development. Higher pressures of oxygen (7 to 8 AA) did inhibit early cleavage; later embryonic development under hyperbaric conditions was not studied. It would be of interest to determine whether or not the delay period in inhibition in our experiments would be shortened by using oxygen pressures higher than 3 AA.

In this species, as in our previous experiments with *Tubularia,* (Miller *et al.,* 1969) hyperbaric oxygen has proven to be a reversible inhibitor of development; it is hoped that our findings may stimulate the use of HBO in a variety of systems as a tool in studies of morphogenesis.

SUMMARY

The effects of hyperbaric oxygen (HBO) upon the embryonic development of *Arbacia* were studied from fertilization to the time of formation of the pluteus larva. Fertilized eggs were incubated in sea water at 16° C in air or in 3 atmospheres absolute (AA) pure oxygen in a hyperbaric chamber. Pressure control experiments using 1 atmosphere oxygen and 2 atmospheres nitrogen demonstrated that the changes observed were caused by elevated pressure of oxygen, and not merely to high ambient pressure. Animals exposed continuously to hyperbaric oxygen for 48 hours were arrested in the gastrula stage; the archenteron

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FIGURE 9. Normal embryos which have developed in air at 16° C for 36 hours, gastrula stage. Compare morphology with that of the inhibited gastrulae shown in Figure 4; whole mount.

FIGURE 10. Normal embryos which have developed in air at 16° C for 48 hours, young plutei. Compare with embryo which has developed for the same period of time at the same temperature, but under 3 AA oxygen, shown in Figure 5; whole mount.

FIGURE 11. Pluteus larvae reared at 16° C under normobaric conditions in air for 72 hours; whole mount.

FIGURE 12. Pluteus larvae reared at 16° C under 3 AA pressure (1 atmosphere oxygen, 2 atmospheres nitrogen) for 72 hours. Plutei are indistinguishable morphologically from embryos reared under normobaric conditions (*cf.* Fig. 11); whole mount.

was observed to form at 30–32 hours, but to regress, resulting in an unorganized mass of cells within the blastocoel. If removed from HBO at 48 hours, over 90% of these inhibited embryos proceeded to form normal prisms and plutei within 144 hours following removal. In respect to reversibility, therefore, HBO differs significantly from many chemical inhibitors of differentiation.

Embryos reared in HBO following development in air for the first 12 hours after fertilization failed to form plutei by 72 hours, whereas controls did so within 48 hours. Embryos in which exposure to HBO was delayed 18 or 24 hours were delayed in reaching the prism or pluteus stage, and showed evidence of disaggregation within 72 hours post-fertilization. Embryos exposed to HBO following 36 hours of development escaped both the inhibitory and lethal effects of HBO and proceeded to form normal plutei by 48 hours. The inactivation by HBO of sulfhydryl-containing enzymes which normally are maximally active during gastrulation in the sea urchin may contribute to the failure of embryos treated with hyperbaric oxygen to complete gastrulation.

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SOME FACTORS CONTROLLING REPRODUCTION IN THE SPIDER CRAB, *LIBINIA EMARGINATA* [1]

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While oogenesis in crustaceans has frequently been studied, detailed knowledge of the anatomy, histology and hormonal controls involved in reproduction in female brachyurans have been limited (see Ryan, 1967; Adiyodi and Adiyodi, 1970 for review). The structure and function of the reproductive system in the crab *Portunus* during the molting and reproductive cycles of the preadult and two adult instars has been examined recently (Ryan, 1967). Although a definite relationship exists between the molting and reproductive cycles in crustacean females, little is known regarding this relationship between molting and sexual maturation among the members of the family Majidae to which *Libinia* belongs. For instance, *Maja* is reported to undergo a final or terminal molt (Drach, 1939). Whether this occurs in all Oxyrhyncha is not known.

This study was undertaken to investigate the relationship between molting and sexual maturation and to determine what if any hormonal controls may be operating in ovarian development in the female spider crab, *Libinia emarginata*.

MATERIALS AND METHODS

Immature and mature female specimens of *Libinia* were collected by the Supply Department of the Marine Biological Laboratory, Woods Hole, Massachusetts during the summers of 1967–1970. The crabs were maintained in aquaria with running sea water and fed periodically on *Mytilus* or *Spisula*. Carapace length of all crabs studied was measured with calipers.

The eyestalks of immature and mature female crabs were removed at their base with a dissecting needle and scissors to induce molting. No special measures were needed to control or prevent excessive bleeding. In addition, mature females with or without broods were subjected to eyestalk ablation and the effects on reproduction were studied. Control animals (non de-eyestalked) were kept in adjacent aquaria. Males were placed in all aquaria.

Reproductive tracts of both immature and mature females were dissected for study. The ovaries were fixed in calcium-formol, dehydrated and embedded in paraffin for light microscopy or were fixed in Karnovsky's (1965) paraformaldehyde-glutaraldehyde fixative. The latter were postfixed in 1% OsO_4, dehydrated

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[1] Contribution no. 211 from the Institute for Molecular and Cellular Evolution, University of Miami. Work was supported in part by NIH grant (5T1-HD-05 to 09) to the Fertilization and Gamete Physiology Training Program at the Marine Biological Laboratory.

[2] NIH Career Development Awardee.

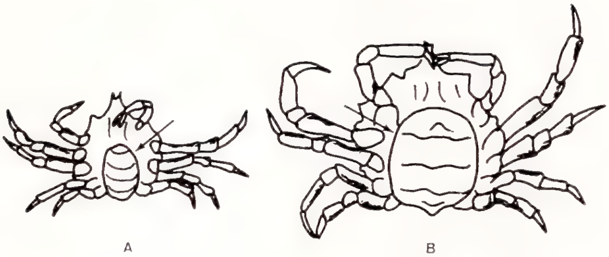


FIGURE 1. Ventral view of immature (A) and mature (B) female *Libinia*. Note difference in outline of abdomen (arrows), × 1/2.

and embedded in Araldite for electron microscopy. Paraffin sections (5 μ) or thick plastic sections (1 μ) were stained with toluidine blue to determine the extent of ovarian differentiation at various stages.

OBSERVATIONS

Sexual maturation and molting

Classification of females as immature or mature was determined on the basis of size and shape of their abdomens. The abdomen of immature females is narrow and does not extend to the base of the legs (Fig. 1). In the adult, it is rounded and almost reaches to the base of the legs (Fig. 1). Figure 2 indicates the carapace length in centimeters of immature and mature females. The maximum length of immature females seen from the wild populations was 6.0 cm. Mature females

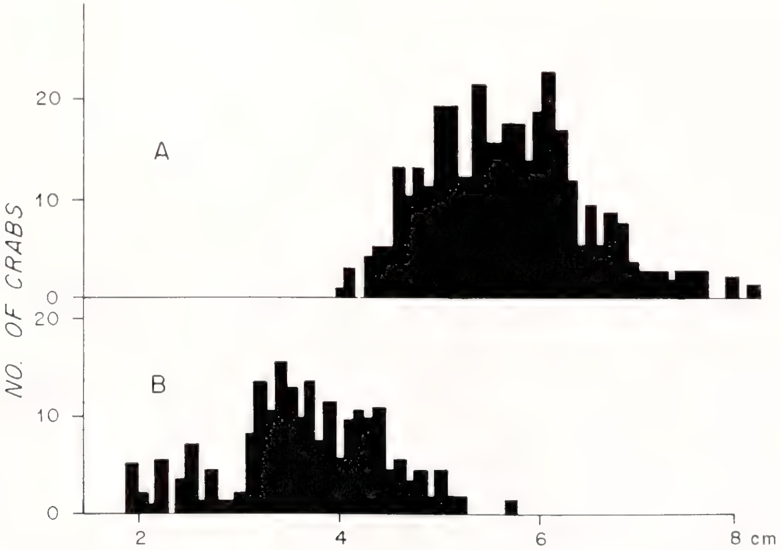


FIGURE 2. Carapace length of individual crabs. A indicates the length of 318 mature females, B—the length of 202 immature females.

exceed 4.0 cm. Thus, in the 4–6 cm range, females may be either mature or immature.

Among the immature spider crabs studied (above 1.9 cm carapace length), molting normally was observed to begin in August or September. The time varied from middle August during the summer of 1968 to the middle of September in 1970. Successful molting occurred even when large numbers of crabs occupied the same aquarium. Carapace length of several immature control females prior to molt and length and sexual state after a normal molt are shown in Figure 3. Crabs undergoing normal ecdysis vary in the amount of growth following the molt as well as the state of sexual maturation. No mature females were ever observed undergoing ecdysis.

Immature and mature females were destalked and observed for indications of molting. None of the 43 mature destalked females molted or upon dissection showed signs of molting. Among the 51 immature females which were destalked in late July or August, most began molting within two weeks after the operation. Those destalked in May or June showed no signs of molting within two weeks. Molt, however, was initiated after four to five weeks. Destalked crabs had difficulty in completing the molting process and although the number of crabs per aquarium was reduced (10 per aquarium), a molting crab frequently was attacked

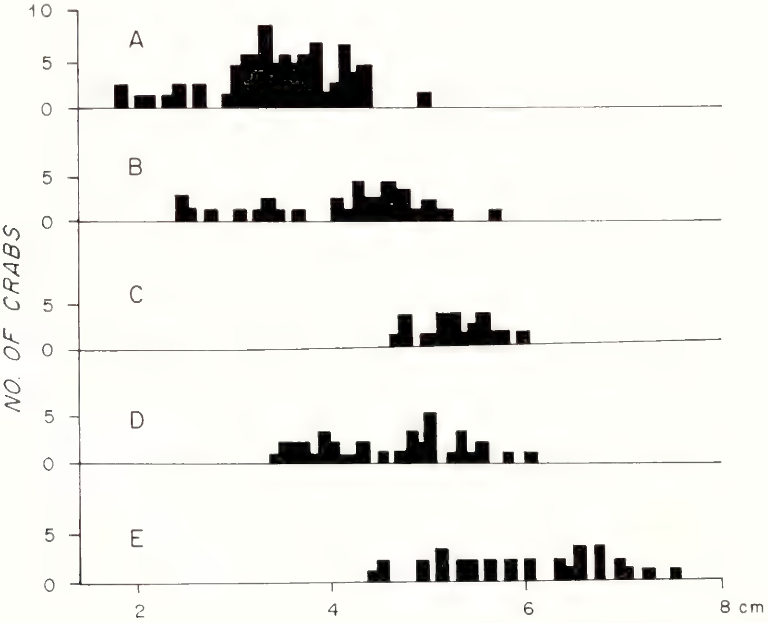


FIGURE 3. A–C, carapace length of intact females; A—carapace length of 76 immature control female crabs, B—Thirty seven females which remained immature following a normal molt, C—Twenty one females which molted to maturity, D–E Destalked immature females. In D, the carapace length (in centimeters) prior to the molt of 38 immature females is indicated. In E, the carapace length of 32 recovered females all of which retained the immature apron.

and eaten by her aquarium mates. Such behavior was not seen in molting, normal, unoperated crabs even under crowded conditions (30–40 per aquarium). In addition, destalked crabs frequently decorated their carapaces with bits of shell, sea urchin spines, and other debris found on the aquarium bottom. Unoperated crabs rarely did such in these experiments.

As reported earlier (Hinsch, 1970), destalked immature females rarely molted to the mature state. This was particularly true when immature females of various sizes were destalked several weeks prior to the time when their controls commenced to molt naturally. Giant immature females were produced. As shown in Figure 3, one might have expected on the basis of carapace length alone (compare with Fig. 2) that several of these crabs would have been mature. However, immature females which were destalked and which molted at the same time as their controls frequently molted to maturity. Two destalked females who had molted once following the operation underwent an additional molt two weeks after the first. This molt was fatal. The abdomen and ovaries of these two crabs were those of an immature female.

TABLE I

Destalking experiments of mature female Libinia. The experimental females were destalked on August 7, 1969 and observed periodically thereafter. Oviposition was stimulated in the operated females

	Egg mass/No. of crabs						
	Aug. 11	Aug. 18	Aug. 26	Sept. 2	Sept. 11	Sept. 15	Sept. 23
Experimentals (destalked)							
20 with eggs in brood pouch	19/19	10/19	14/16	14/15	6/15	9/15	10/15
16 without eggs in brood pouch	0/14	0/14	11/12	9/12	5/12	8/11	9/9
Controls							
15 (eyestalks not removed)	2/15	3/14	3/13	1/14	0/14	0/14	0/14

Reproductive cycles

The breeding behavior and reproductive cycles of the female *Libinia* have been described (Hinsch, 1968). Mature females apparently oviposit for the first time in late May or early June. This brood is often quite small. It is followed by additional spawnings at 25 days intervals. The time of last oviposition varies seasonally.

To determine the role of the eyestalk hormones on reproduction, mature females were destalked during various stages of their ovarian cycles. Following the operation, the crabs were placed in aquaria with some males and observed. The 25 day brood period was unaltered and the females continued to attract males at the time of zoeae hatching as did those in the control tanks (Hinsch, 1968).

In July 1968, 43 mature females with broods were destalked. On September 23, 24 of the 27 survivors had orange egg masses (new). Among the wild population, only 3 of 93 mature females carried broods at this time.

In early August 1969, mature females with broods and females who had been without broods for at least one week were subjected to destalking and observed periodically (Table I). By late September, only females which had been destalked carried egg masses in their brood pouches.

On September 3, 1970, these experiments were repeated on an additional 130 crabs. Seventy females with broods, many of them relatively new (orange egg masses), were used as controls. Sixty females lacking broods were separated into two groups. Thirty were left unoperated and placed in an aquarium with males. The other thirty were destalked prior to being placed with males in another aquarium. All crabs were observed periodically and the presence or absence of a brood noted (Table II). The operated females were frequently observed mating with males, while unoperated females rarely mated at this time of the year. On September 26, 27 of the 28 surviving operated females contained orange (new) egg masses in their brood pouches. Of the 99 unoperated females, 5 had new (orange) egg masses, 5 had brown egg masses containing zoeae about to hatch and 88 were without egg masses. As noted earlier (Hinsch, 1968) a female need not mate immediately before each brood of eggs is laid to produce viable young.

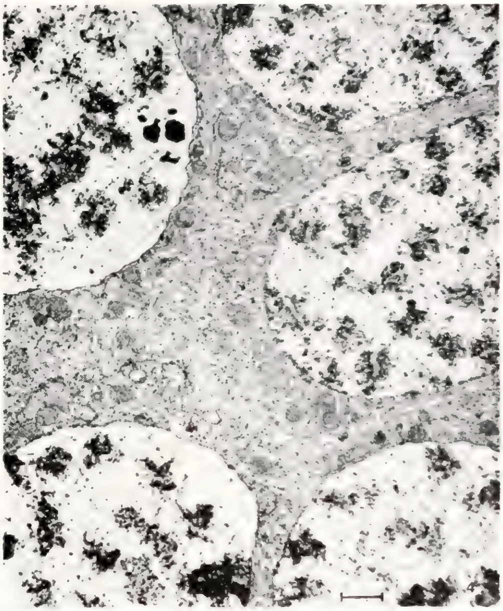
TABLE II

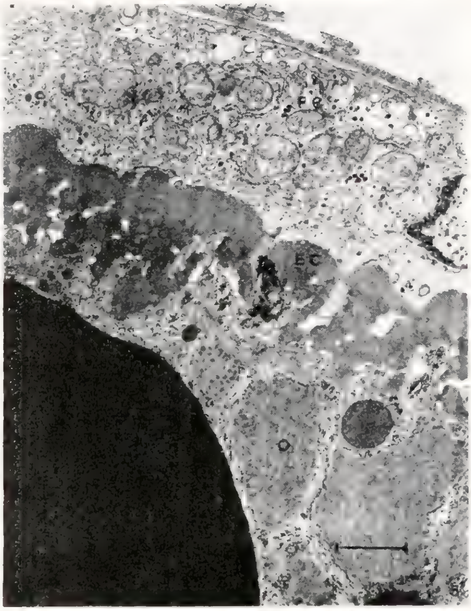
Presence or absence of egg masses in the brood pouches was observed in mature females. As indicated, oviposition was stimulated in the females which have been destalked

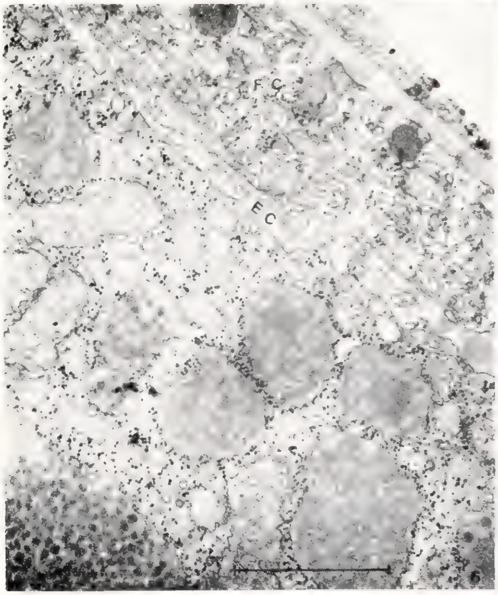
(1970)	Egg masses/No. of crabs		
	Sept. 3	Sept. 21	Sept. 26
Experimental females (destalked)	0/30	14/29	27/28
Control females (non-destalked)			
Females with broods	70/70	12/70	10/70
Females without broods	0/30	1/29	1/29

Ovarian development

Dissection of immature females revealed ovaries which were small, white H-shaped organs. Sections of these ovaries showed large masses of immature oocytes near a central core of syncytial cells and showed no signs of vitellogenesis (Fig. 4). The ovaries of females which have just molted to maturity are also white and only slightly larger than those of immature females. These oocytes are small, have vacuolated nucleoli and no yolk. One month following the molt to maturity, many of the crabs have ovaries which are enlarged, orange in color and have oocytes in which vitellogenesis is well advanced. Following oviposition, ovarian development takes place in the ovary of the mature female as she is brooding her young. Eggs collected near the end of a brooding cycle have well developed yolk and a forming egg coat (Fig. 5). Females who are about to release zoeae during the breeding season have oocytes in their ovaries which are fully developed, surrounded by an egg envelope and lack follicle cells. As each brood hatches, a new mass of eggs is generally oviposited after approximately 6 to 12 hours (Hinsch, 1968). Ovaries of females at the end of the breeding season vary in stage of maturation, although







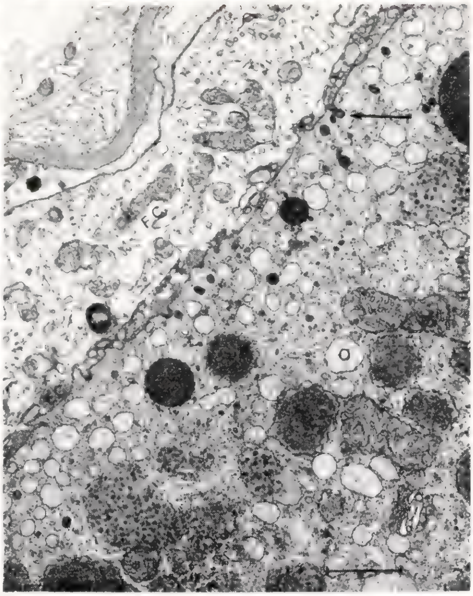


FIGURE 4. Ovary from an immature female collected in mid-August. Premolt condition is unknown. Many small oocytes are apparent.

FIGURE 5. Oocyte from mature female brooding young. The oocyte (O) is surrounded by forming egg coat (EC) and follicle cells (FC).

FIGURE 6. Mature oocytes from bright orange ovary of female at the end of the breeding season. Follicle cells (FC) still surround the oocyte and egg coat (EC) has only partially formed.

FIGURE 7. Oocyte from orange ovary of a mature female in mid-November. In this instance vitellogenesis including pinocytosis (arrow) is seen and beginnings of egg coat (EC) are seen between oocyte (O) and follicle cells (FC). Whether this female had recently molted to maturity is not known.

most are usually bright orange and contain large oocytes filled with yolk (Figs. 6 and 7). In many cases these oocytes are still surrounded by follicle cells and may or may not show traces of an egg envelope (Figs. 6 and 7). Thus, these oocytes apparently overwinter until the next breeding season commences the following spring and are released as the first brood of the year. Mature females shipped to Florida from Woods Hole during the winter months have well developed ovaries and some days after acclimatizing to the warmer water temperature oviposit.

Seminal receptacles are absent in the immature females and very small in those newly molted to adulthood. In addition, newly molted adult females have not been observed mating in the soft condition.

Discussion

Arthropod metamorphosis, particularly phases of molting and sexual maturation, have been extensively studied in recent years. It is particularly well documented in the insects. Although crustaceans have been studied less extensively, many similarities to the insects have been noted. Ecdysis is controlled by neurosection from the X-organ (crustaceans) of the eyestalk or protocerebrum (insects). This neurosecretion activates the Y-organ (crustaceans) or corpora cardiaca (insects) to produce a hormone which initiates molting. Molting can be initiated in crustaceans by the removal of the eyestalk with its contained X-organ-sinus gland complex (See Passano, 1960; Highnam and Hill, 1969; Adiyodi and Adiyodi, 1970 for reviews). In *Libinia* the duration of time between eyestalk ablation and onset of molting varies and seems dependent on the relationship of time of ordinary ecdysis. Further factors may include temperature changes, nutrition, salinity and light-darkness periods as have been suggested by Aiken (1969) and Stephens (1952). Immature *Libinia* females molt following eyestalk ablation although mature crabs never have been seen to molt after reaching maturity. Thus *Libinia* like *Maja* (Drach, 1939) apparently undergoes a terminal molt to maturity. Perhaps this may be true of all members of the family Maijdae. In *Maja* (Carlisle and Knowles, 1957) failure to molt beyond the terminal stage has been attributed to the degeneration of the Y-organ. Whether the Y-organ degenerates in *Libinia* is not known.

Sex differentiation in crustaceans as in most animals is genetically determined although hormonally mediated. Sexual differentiation of the male is apparently controlled by the androgenic gland (Charniaux-Cotton, 1964; Charniaux-Cotton *et al.*, 1966). Females develop in the absence of the androgenic substance secreted by this gland. They can be masculinized by transplantation of the androgenic gland to their bodies. In most crustaceans, the reproductive cycles and body growth cycles are closely related and regulated by interlinked control mechanisms. These include the eyestalk hormones which control molting, oogenesis, vitellogenesis and secretion of sex hormones (for review see Adiyodi and Adiyodi, 1970) in many crustaceans having preadult and multiple adult female instars. Reproductive hormones influence the synthesis and accumulation of a sex-related lipoprotein utilized in vitellogenesis (Adiyodi, 1968a, b). Such a lipoprotein is found in mature female *Libinia* undergoing vitellogenesis but is absent in the blood of immature females and males (Bischoff and Telfer, University of Cincinnati and University of Pennsylvania, unpublished).

In *Libinia* sexual maturation of the females apparently occurs only following the terminal molt. Reproduction in this form can thus be studied independently of molting and the factors controlling sexual maturation recognized. Evidence presented here suggests some of the parameters of endocrine control of reproduction in *Libinia*. The eyestalk hormone apparently does not act to inhibit all stages of vitellogenesis. Evidence for this is the mature ovaries found in females out of the breeding season. In addition, the eyestalk hormone(s) seem to govern or regulate egg release. This is indicated by the continued oogenesis and oviposition beyond the normal season or resumption of oviposition following eyestalk ablation. The time delay between eyestalk removal and oviposition suggests that yet another factor which is controlled by the eyestalk hormone(s) may be involved. This could be a stimulating substance produced by the neurosecretory cells of the cerebral and/or thoracic ganglia (Ōtsu, 1963; Parameswaran, 1955) or could simply be the time needed for metabolic breakdown of the inhibiting hormone following eyestalk ablation. The fact that destalked immature females do not molt to maturity also implies hormonal control from a source other than the X-organ-sinus gland complex. The exact nature and origin of such factor(s) is yet to be determined. In some crustaceans the Y-organ has been suggested as a source of a gonad-maturation factor. However, if in *Libinia* the Y-organ degenerates as in *Maja* following the molt to maturity, then one must rule out such a role and look elsewhere.

Although the soft conditions following molting is generally considered favorable for mating in crustaceans it may be unimportant in *Libinia* since mating can occur in a hardened female (Hinsch, 1968). As reported here, newly molted mature females have not been observed to mate although such may occur under natural conditions. Males have not been seen carrying premolt females about as happens in other species, *e.g., Portunus* (Ryan, 1967), *Maja* (Schöne, 1968). The small size of the seminal receptacle in the newly molted adult *Libinia* suggests that the initial mating may occur much later in the hardened female. Hartnoll (1963) has reported that female *Maja* and *Pisa,* species belonging to the same superfamily as *Libinia,* are physically incapable of mating prior to the molt to puberty. This is the terminal molt. In general, ovarian maturation in members of this superfamily (*i.e., Pisa, Inachus, Hyas*) occurs after the molt to maturity (Hartnoll, 1963) as we have found in *Libinia.*

SUMMARY

1. Carapace length is not sufficient for determining state of sexual maturation of female *Libinia.* Females in range of 4–6 cm carapace length may be mature or immature. Shape of the abdomen distinguishes between mature and immature females.

2. Eyesalk ablation of mature female *Libinia* results in extended periods of or initiation of the ovigerous state but does not appear to initiate molting. Breeding behavior and reproductve cycles seem unaltered by destalking.

3. Immature female *Libinia* which have had their eyestalks removed molt precociously but rarely to maturity.

4. *Libinia* apparently undergo a terminal molt to maturity. Ovarian development and vitellogenesis occur only in mature females.

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SUGAR RELEASE AND PENETRATION IN INSECT FAT BODY: RELATIONS TO REGULATION OF HAEMOLYMPH TREHALOSE IN DEVELOPING STAGES OF *HYALOPHORA CECROPIA*

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In insects, the fat body is the chief site of synthesis of the disaccharide trehalose, which is generally the predominant circulating sugar (review: Wyatt, 1967). There is evidence for homeostatic regulation of haemolymph trehalose levels (Saito, 1963; Friedman, 1967; Wyatt, 1967; Nettles, Parro, Sharbaugh and Mangum, 1971) and, to account for this, feedback mechanisms have been proposed. While inhibition and activation of enzymes concerned with trehalose synthesis have been demonstrated *in vitro* (Murphy and Wyatt, 1965; Friedman, 1968), the mechanisms responsible for regulation *in vivo* are unclear. One hitherto unanswered question is how an insect can maintain its haemolymph sugar at distinctly different levels in its different developmental stages, as, for example, the cecropia silkmoth has been shown to do (Wyatt, 1967).

The dynamic relations between intracellular and extracellular trehalose are clearly important for the regulation of trehalose synthesis, yet very little is known about these. Mochnacka and Petryszyn (1959) found trehalose to be higher in haemolymph than in "bled tissues" of pupae of the sphingid *Celerio euphorbiae,* and larval and adult tissues of this species apparently contained no trehalose. Fat body of cecropia silkmoth larvae (Wyatt, 1967, page 297) and wax moth larvae (Lenartowicz, Zaluska and Niemierko, 1967), after rinsing in saline solutions, was found to contain trehalose at levels much lower than those in the haemolymph of the respective insects. These reports suggest that movement of trehalose from fat body to haemolymph might require active transport, albeit active transport of sugars has never been demonstrated in an insect. In fact, the absorption of sugars from the insect gut, in contrast to analogous processes in vertebrates, depends upon simple diffusion (Wyatt, 1967).

Fat body of larval blowflies (*Phormia regina*), on the other hand, contained trehalose even though none could be found in the haemolymph of the same stage (Wimer, 1969).

In view of these problems, we have examined the relations between fat body and haemolymph sugar in several insect species. We find that fat body analyzed without prior rinsing may contain sugar at levels lower than, equal to, or higher than those in the haemolymph, but during rinsing in isosmotic media much of the tissue sugar is rapidly released. During ontogeny of the cecropia silkmoth, the quantitative relationships between haemolymph and fat body trehalose change markedly. Correlated with these changes are alterations in fat body function with respect to release of endogenous and penetration of exogenous trehalose, which we interpret as reflecting changes in the cell membrane having regulatory significance.

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MATERIALS AND METHODS

Animals and media

Most of the experiments were conducted with cecropia silkmoths (*Hyalophora cecropia*), reared from genetically mixed stock either outdoors on wild cherry foliage or in the laboratory at 25° C on an artifical diet (Riddiford, 1968) under 16 hours light per day. After spinning their cocoons, outdoor reared animals were held at 25° C for 2–4 months and then (a) chilled at 6° C to activate the neuroendocrine system for development, or (b) debrained to establish permanent diapause (Williams, 1946) and then kept at 15° C, or (c) left at 25° C, at which temperature diapause persisted in most individuals for several months. In many instances, pupal respiration was measured, and the criterion for diapause was an O_2 consumption of less than 20 µl per gram live weight per hour (Schneiderman and Williams, 1953). Pharate adults ("developing adults") were obtained by incubating previously chilled pupae at 25° C, and stages were recognized as described by Schneiderman and Williams (1954). Insects of other species, used in a few experiments, were obtained as described in the footnotes to Table I.

Injections were made through the thoracic tergum of pupae in volumes not more than 50 µl, and between the first pair of dorsal tubercles of larvae in not more than 100 µl. Experimental injury consisted of making 20 punctures with a 25 gauge needle in the thoracic tergum, which were then sealed with melted paraffin wax. Haemolymph was collected at the time of injury by puncturing a wing sac with the tip of a scalpel and then sealing the hole. When haemolymph was collected for use in media, a few crystals of phenylthiourea were added to prevent darkening due to the action of tyrosinase.

The media used for rinsing and incubation of tissue from all insects except *Blaberus discoidalis* were: (i) that of Reddy and Wyatt (1967) (RW medium) containing NaCl 20 mM, KCl 80 mM, $CaCl_2$ 4 mM, $MgCl_2$ 15 mM, phosphate 8 mM, and 20 amino acids (total 91 mM), the sugars in the published medium being omitted, with pH 6.5 and osmotic pressure approximately 450 milliosmolar; (ii) modified RW medium in which the concentrations of NaCl and KCl were reduced to 4 and 50 mM, respectively (total 360 milliosmolar); and (iii) high K^+-Mg^{++} lepidopteran saline, modified after Weevers (1966) and Pan, Bell and Telfer (1969), which contained NaCl 4 mM, KCl 140 mM, $CaCl_2$ 4 mM and $MgCl_2$ 15 mM (total 350 milliosmolar). In initial experiments RW medium was used, while later the modified RW medium was substituted, and no differences were observed in the results. For *B. discoidalis,* a medium was used consisting of NaCl 130 mM, KCl 10.3 mM, $CaCl_2$ 4.5 mM, $MgCl_2$ 8 mM, and phosphate buffer 5 mM, together with the amino acid mixture of RW medium (pH 6.5, 425 milliosmolar; *cf.* Van Asperen and Van Esch, 1956).

The sugar content of haemolymph and fat body, and efflux from fat body in vitro

Haemolymph and fat body were taken from individual insects for analysis. The fat body was dissected out, blotted by drawing it repeatedly across Glassine weighing paper (Eli Lilly and Company, Indianapolis, Indiana), and portions of 50–500 mg were homogenized for analysis immediately, or after immediate freezing, or after incubation at 25° in not less than 1.8 ml of medium. For analysis, blotted tissue samples were weighed to the nearest milligram on a torsion balance and ground in glass homogenizers in three volumes of 6.7% trichloracetic acid. Homogenates were transferred to 0.5 ml capped polyethylene tubes, and centrifuged at 8000 × *g* in a Microfuge (Model 152; Beckman Instruments, Palo Alto, California). Ten-microliter samples of the clear supernatant were transferred into 0.29 ml of 95% ethanol to precipitate the glycogen, and recentrifuged. Samples (150 microliters) for determination of trehalose were then taken and evaporated to dryness below 60° C in a vacuum oven, and 1.20 ml of fresh anthrone reagent (Mokrasch, 1954), with increased water to allow for the use of dry samples, was added to each. The tubes were heated in a boiling water bath for 5 minutes, then cooled in ice and absorbance was determined at 620 nm with a Zeiss spectrophotometer. Haemolymph trehalose was measured by treating 1–10 microliters of whole haemolymph (which contains negligible ethanol-precipitable carbohydrate) with anthrone reagent as just described. Glucose standards were run simultaneously.

The sugar measured by the anthrone reaction is designated as trehalose. In haemolymph of the cecropia silkmoth, trehalose has been identified by specific methods and only minute amounts of other sugars are present (Wyatt and Kalf, 1957; Wyatt, 1967). We tested an extract of fat body from diapausing cecropia pupae for reactivity with glucose oxidase with and without prior treatment with trehalase (prepared from *Tenebrio molitor* larvae) and found abundant trehalose but no detectable free glucose. The assumption that trehalose is the significant sugar in our experiments with *H. cecropia* therefore seems justified. In the other insect species, we have not established what proportion of the anthrone-reactive sugar is trehalose. In the results that follow, data obtained by the anthrone reaction are expressed in the Figures and Tables as glucose equivalents, while in the text, sugar is assumed to be trehalose and is expressed on a molar or molal basis as such (= glucose equivalents × 0.5).

The amount of adhering haemolymph in a number of fat body tissue samples from cecropia pupae was determined by marking the haemolymph with ^{14}C-inulin, injected 3 hours before the animals were dissected, as described by Cherbas and Cherbas (1970). The content of haemolymph in blotted fat body was found to be 20–40%, the majority of samples having 30–35%. In most experiments, haemolymph contamination was not measured, but was assumed to be 30%; thus, cellular volume was estimated as blotted tissue weight × 0.7, the specific gravity being taken as 1.0. Underestimating haemolymph contamination in the tissue samples would minimize the true differences between trehalose concentrations in the cells and in the haemolymph.

Concentrations of sugar in haemolymph can be expressed in molarity, based on volume, but to permit osmotic comparison with the intracellular fluid it was desirable to express them also in molality. For this purpose, 100 ml of haemolymph was assumed to contain 85 g of water, a mean value appropriate for cecropia pupal haemolymph, though for larval blood the true water content is probably somewhat higher (Wyatt and Kalf, 1957). The water content of fat body cells was estimated by weighing portions of the tissue before and after exhaustive drying, correction being made for extracellular fluid with the aid of radioactive inulin. Based on several measurements on tissue from fully-grown feeding larvae and from diapausing pupae of cecropia, the following percentages of cellular fresh weight were assumed for intracellular water: larval fat body, 50%; pupal fat body, 70%. With other insect species, the value of 50% was used for fat body from the larval or adult stages, and 70% for pupal tissue.

Treatment of data

For a given treatment, the concentration of trehalose in the fat body was frequently found to bear a constant relationship to that in the haemolymph, while the absolute levels varied considerably among individuals of a group. Consequently, the data were normalized by multiplying all individual values for fat body trehalose by the ratio: group mean concentration in haemolymph/individual concentration in haemolymph. This method is comparable to expressing individual concentrations as percentages of haemolymph trehalose, but also permits comparison of the levels of sugar in different batches of animals at the same or different stages of development.

The sexes of the animals used were noted, but no differences related to sex were observed in haemolymph trehalose, fat body trehalose, or the effect of any treatment.

Uptake of ^{14}C-solutes

Uptake of 1-^{14}C-glucose, U-^{14}C-glycerol and 6-^{14}C-trehalose into fat body *in vitro* was measured with the aid of ^3H-inulin as a marker for extracellular fluid. Ten microliters of a stock solution containing 2×10^4 counts/min per microliter each of ^{14}C-solute and ^3IL-inulin were added to 1.00 ml of RW medium in a 10 ml beaker. A 10 μl sample of this mixture was taken for determination of the initial $^{14}C/^3H$ ratio. Fat body (0.2–1.0 g, pooled from 2–5 animals) was added, continuous shaking was commenced, and 5 μl samples of the medium were taken approximately each minute for 10 minutes. Difficulty was experienced from clogging of the micropipets by fragments of tissue, and toward the end of this work a vessel was constructed containing a suspended well bottomed with 100-μm nylon mesh which provided a pool of tissue-free medium. This allowed more frequent and accurate sampling. The samples were mixed with 0.1–0.2 ml of NCS (Amersham-Searle, Des Plaines, Illinois) to which 5 or 10 ml of toluene phosphor scintillation fluid was then added. Radioactivity was counted in a Packard scintillation counter.

Because of the difficulty in obtaining precisely measured samples of medium, calculations were based on isotope ratios, which are independent of sample size. The initial ratio of ^{14}C-solute/^3H-inulin (R_0) represented no penetration of solute, while a calculated theoretical final ratio (R_x) represented equal distribution of ^{14}C-solute in fat body intracellular water and the incubation medium (see Equation 1). From the observed ratio at any time (R_t), the percentage penetration could be calculated (Equation 2). The $^{14}C/^3H$ ratio generally became essentially constant within 10 minutes of incubation, and in the case of diapausing pupal fat body did not change appreciably during an additional 50 minutes of incubation.

$$R_{\infty} = \frac{m + hs}{m + hs + (1-h)ds} \times R_0 \qquad (1)$$

Inserting values of 0.3 for h and 0.5 or 0.7, respectively, for d,

$$R_{\infty} = \frac{m + 0.3s}{m + 0.65 \text{ (or } 0.79)s} \times R_0 \qquad (1a)$$

$$\text{Percentage of penetration of solute} = \frac{R_0 - R_t}{R_0 - R_{\infty}} \times 100 \qquad (2)$$

Where m = volume of incubation medium (1.0 ml), s = volume of tissue sample, assuming 1 g = 1 ml, h = fraction of haemolymph in tissue sample (generally assumed to be 0.3), d = fraction of water in fat body cells (assumed to be 0.5 for larval tissue or 0.7 for pupal tissue), R_0 = initial ratio of $^{14}C/^{3}H$ in the medium, R_{∞} = theoretical final ratio of $^{14}C/^{3}H$, assuming complete penetration of cell water by ^{14}C-solute and no penetration by ^{3}H-inulin, and R_t = observed ratio of $^{14}C/^{3}H$ after incubation.

RESULTS

The sugar content of fat body and haemolymph of insects at different stages of development

The levels of anthrone-reactive sugar, which in cecropia silkmoths will be regarded as trehalose (see Materials and Methods) were measured in unrinsed fat body (corrected for measured or estimated adhering haemolymph) from several developmental stages of four insect species belonging to three orders (Table I). The relationships between sugar in haemolymph and in fat body were fairly constant among individuals of the same species and stage of development, but showed substantial variation in different species and stages. Thus, fat body sugar was significantly higher than haemolymph sugar in adults of *Blaberus*, about equal to haemolymph sugar in *Phyllophaga* larvae, and lower than haemolymph sugar in *Manduca* larvae and pupae. In *Hyalophora cercopia*, as previously observed (Bade and Wyatt, 1962; Wyatt, 1967), the level of trehalose in the haemolymph changed markedly in different developmental stages, being 40–50 mM in the fully-grown larva, falling to 8–15 mM in the diapausing pupa, and rising again to 30–40 mM in the pharate adult. Intracellullar sugar underwent less extreme change, and was found in the fat body of larvae and early pharate adults at nearly their respective haemolymph levels and in that of pupae at more than twice their haemolymph level. Comparable changes between developmental stages were not observed in *Manduca sexta*.

When fat body from the cockroach *B. discoidalis* or larvae, pupae or pharate adults of the cecropia silkmoth was incubated briefly in culture media (see Materials and Methods), a significant proportion of its sugar content escaped (Tables I, II) Since this phenomenon appeared to represent a potential source of serious misconception arising from analysis of rinsed tissues, and further might have physiological importance, we decided to investigate it further.

TABLE I

Sugar concentrations in haemolymph and fat body from various insects. Portions of fat body were incubated for 10 minutes in RW medium (or Blaberus *medium) with or without 2% bovine serum albumin (BSA). Sugar was determined by anthrone, and concentrations are expressed in molalities as glucose equivalents in haemolymph or fat body water ± S.E.M. The results for fat body were corrected for adhering haemolymph or medium, and the water content of haemolymph and fat body was estimated, as described in Materials and Methods*

Order	Dictyoptera	Coleoptera	Lepidoptera					
Species	*Blaberus discoidalis**	*Phyllophaga species***	*Manduca sexta****		*Hyalophora cecropia*			
Stage	Adults	Larvae	Larvae	Diapause pupae	Larvae†	Larvae††	Diapause pupae‡	Pharate adults, 2–3 day ‡‡
Number of animals	4	3	4	3	2	22	5	4
Sugar concentrations (as glucose)								
Haemolymph (millimolar)	49.1 ± 4.9	68.2 ± 3.6	71.0 ± 8.9	80.7 ± 5.3	86.7 ± 34.0	95.2 ± 3.2	16.7 ± 0.8	59.6 ± 3.8
Haemolymph (millimolal)	57.8 ± 5.8	80.2 ± 4.2	83.5 ± 10.5	94.9 ± 6.2	102.1 ± 40.0	112.0 ± 3.8	19.6 ± 0.9	70.1 ± 4.5
Fat body, unrinsed (millimolal)	107.2 ± 16.2	86.9 ± 3.1	46.6 ± 5.4	57.1 ± 6.2	110.3 ± 11.8	84.8 ± 3.3	41.4 ± 2.7	84.7 ± 6.7
Fat body, incubated (millimolal)								
Protein-free medium	71.8 ± 5.7				46.7 ± 8.0			44.2 ± 0.9
Medium + 2% BSA	80.2 ± 7.3				54.7 ± 8.3			55.4 ± 6.7
Sugar molality ratio, unrinsed fat body/haemolymph	1.85	1.08	0.56	0.60	1.08	0.76	2.11	1.21
Retention in fat body in medium without protein (%)	67				42			52
Retention in fat body in medium with protein (%)	75				50			65

* Reared at 25° C on corn meal and Purina rat chow.

**Fully grown larvae collected from leaf mold in early May and stored for 2 weeks at 15° C before analysis.

*** Reared at 25° C on artificial diet (Yamamoto, 1968) on a light regime of 12L/12D. The larvae were taken on the day of burrowing and the pupae 2 months after pupation.

† Laboratory reared as described in Methods, taken 1 day before spinning.

†† Outdoor reared, acclimatized at 25° C, 0–4 days before spinning.

‡ Outdoor reared, held for 3 months at 25° C after spinning.

‡‡ Outdoor reared, chilled for 4 months at 6° C, then returned to 25° C for development.

TABLE II

Concentrations of trehalose in haemolymph and fat body of various batches of cecropia pupae, and the effects of different media on retention of trehalose in fat body. Batches I–III were reared out of doors on foliage and were collected as fresh cocoons on different dates: batch I, June 22, 1970, batch II, July 19, batch III, August 26. Batch IV was reared in the laboratory on artificial diet and spun cocoons in February, 1971. When respiration was measured, only pupae using less than 20 µl O_2 per gram-hour (characteristic of diapause) were used. The haemolymph content of fat body samples was measured (see Methods) in Group A, and was assumed to be 30% in all other groups. Other particulars were as in Table I

	A	B	C	D	E	F	G	H
	Unchilled diapause pupae					Debrained diapause pupae	Pupae chilled 2.5 months	Debrained pupae 6 days after injury*
Batch number, and age of pupae when used (months)	I (4)	I (4)	II (4)	III, IV (3–4)	III (4.5)	III (8)	IV (3)	II (7)
Number of experiments,** and total number of animals	2, 7	2, 6	2, 7	3, 8	2, 6	2, 4	1, 3	1, 3
Respiration measured	No	No	Yes	Yes	Yes	Yes	No	Yes
Sugar concentrations								
Haemolymph (millimolar)	19.0 ± 2.4	14.8 ± 2.1	13.2 ± 0.8	32.3 ± 3.5	27.6 ± 1.9	33.1 ± 4.2	42.6 ± 2.0	39.1 ± 3.0
Haemolymph (millimolal)	22.4 ± 2.8	17.4 ± 2.5	15.5 ± 0.9	38.0 ± 4.1	32.5 ± 2.2	38.9 ± 4.9	50.1 ± 2.4	46.0 ± 3.5
Fat body, unrinsed (millimolal)	42.4 ± 2.1	40.4 ± 2.6	38.0 ± 2.8	40.7 ± 3.2	36.8 ± 3.3	28.8 ± 3.1	37.2 ± 2.7	55.0 ± 2.5
Fat body, incubated (millimolal)								
RW medium	17.3 ± 2.4	16.3 ± 2.4	19.0 ± 0.8	22.3 ± 1.7(5)***	20.2 ± 0.9	19.6 ± 2.9		29.8 ± 5.3
RW + 2% haemolymph protein							31.6 ± 2.4	
RW + 2% BSA			33.9 ± 3.8	36.4 ± 2.4	23.8 ± 1.0	25.1 ± 3.6		28.6 ± 4.6
RW + 2% PVP				34.0 ± 6.1(3)***	19.9 ± 1.9	23.4 ± 3.8		
K-Mg ringer + 2% haemolymph protein							17.3 ± 1.7	
K-Mg ringer + 2% BSA				22.0 ± 1.5	20.8 ± 2.2	21.4 ± 3.4		
Sugar molality ratio, fat body/haemolymph	1.89	2.32	2.45	1.07	1.13	0.74	0.74	1.20
Retention in fat body in RW medium (%)	41	40	50	55	55	68		54
Retention in fat body in RW + protein (%)			89	89	65	74	85	52

* Experimental injury consisted of multiple punctures, as described in Materials and Methods. The mean haemolymph sugar in the same pupae before injury was 24.9 ± 1.3 mM.

** Two to four animals were used in each experiment.

*** Numbers of animals used are in parentheses.

Release of trehalose from cecropia fat body in vitro

Pupae in diapause. A series of experiments was carried out with fat body from cecropia pupae in diapause, in which the initial sugar level was generally about double that in the haemolymph (Tables I, II). During incubation in RW medium, up to 60% of the intracellular trehalose was lost, most of it during the first 2 minutes (Fig. 1; Table II, columns A and B). This release of sugar from the tissue was not a consequence of cell lysis, for a maximum of 4% lysis occurred, based upon the release into the medium of glycogen (measured with anthrone after precipitation with ethanol) or upon the previously established retention of capacity for protein synthesis by fat body in a similar medium (Stevenson and Wyatt, 1962). In this and subsequent experiments (see Fig. 2), the rapid initial loss of a certain fraction of the fat body sugar was followed by little or no further loss during incubation for times up to 30 minutes. The virtual cessation of release could not be due to attainment of diffusion equilibrium, for the volume of medium used was always sufficient to maintain a substantial concentration gradient of sugar toward the exterior (final ratio = 18:1 in the experiment of Fig. 1). These observations are most readily interpreted in terms of intracellular compartmentation: fat body trehalose appears to be divided between rapidly exchangeable and slowly exchangeable compartments.

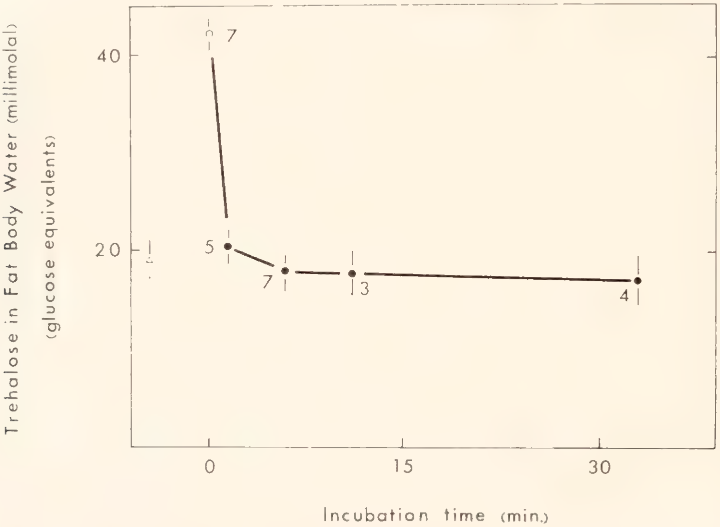

FIGURE 1. Release of trehalose from fat body of diapausing cecropia pupae during 30 minutes incubation in RW medium. Fat body from 4-month-old diapausing pupae (Batch I of Table II) was divided into 4 or 5 portions of 0.1–0.3 g from each pupa, which were separately incubated in 5 ml of RW medium for different times and then taken for analysis; ○, unrinsed fat body; ●, rinsed in RW medium; △, level in hemolymph. Vertical limits are standard errors of the means, and numbers are the numbers of animals used.

Since release of trehalose occurred rapidly upon transfer of tissue from its natural milieu of haemolymph to a medium which, while formulated to resemble haemolymph, lacked protein, we suspected a role of protein in preventing sugar release. We therefore tested the effect of adding cecropia pupal haeolymph protein to RW medium. In the experiment shown in Figure 2, the effects of two levels of protein were tested by incubating tissue for 5 minutes in medium containing 10% protein, which was then diluted with additional medium to 5% and incubation was continued. In the presence of either level of protein, 85–95% of the trehalose in the fat body was retained, compared to only 40% in the protein-free medium. Similar results are shown in Table II (groups C and D).

To determine whether a non-specific protein would also be effective in preventing the release of trehalose from pupal fat body, we tested bovine serum albumin (BSA; Fraction V Powder, Sigma Chemical Corp., St. Louis, Missouri) at several concentrations. The results (Tables II, III) show that, at concentrations above 1%, BSA was as effective as cecropia haemolymph protein. Further evidence for non-specificity of the effect was obtained in experiments with polyvinylpyrrolidone (PVP), a synthetic polymer of approximate M.W. 25,000. PVP was about as effective as BSA, causing (in the experiment of Table II, Column D) the retention of 84% of the initial trehalose, compared with 89% in the presence of BSA. Since it was of further interest to know whether a simple

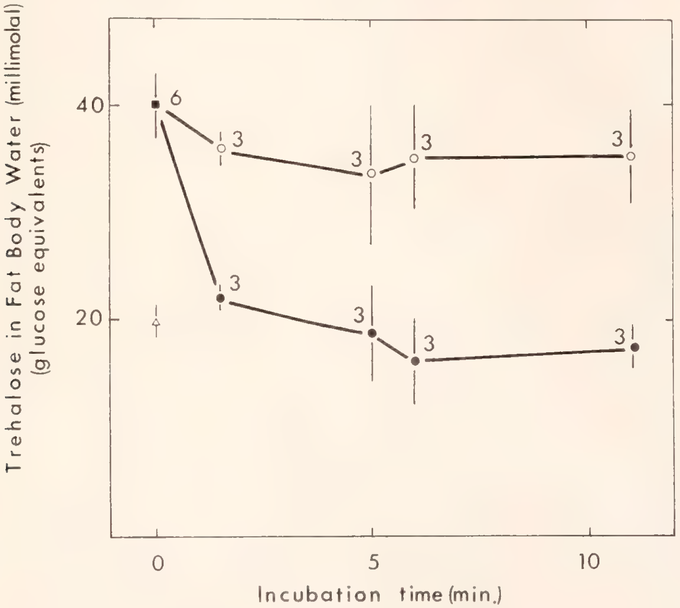

FIGURE 2. The ability of cecropia haemolymph protein to prevent the release of sugar from pupal fat body in RW medium. Experimental details were as in Figure 1, except that one set of fat body samples was placed in RW medium containing 10% pupal haemolymph protein; after 5 minutes, by addition of more medium, the protein was diluted to 5%. The protein was obtained by dialysis of haemolymph from diapausing pupae against 0.01 M phosphate buffer (pH 6.5), followed by lyophilization; ■, unrinsed; ●, RW medium without protein; ○, medium containing protein; △, the level in hemolymph.

salt solution containing protein would suffice to maintain trehalose in pupal fat body, a high K$^+$-Mg$^+$ saline containing 2% BSA was also included in this experiment. In this medium, however, trehalose was released to the same extent as in RW medium *without* protein. The predominant difference between RW medium and the saline was the presence of almost 100 mM amino acids in the former, and we infer that the presence of these, together with a macromolecule, is in some way necessary for maintenance of the sugar content of pupal fat body cells. RW medium also contains 8 mM phosphate, which is lacking from the saline, but addition of phosphate to the saline plus BSA did enhance retention of trehalose.

Larvae. In contrast to the situation in pupae, the concentration of trehalose in unrinsed fat body of fifth instar cecropia larvae was somewhat less than that in haemolymph of the same stage (Table I). Further, when larval fat body was incubated for 10 minutes in RW medium either with or without 2% BSA, 50–60% of intracellular trehalose was lost (Table I). Evidently, larval fat body cells were capable of only partial retention of their sugar even in media containing protein, and the tissue acquires the capacity for nearly complete retention during ontogeny in the transition from larva to pupa. In protein-free media, however, similar fractions of intracellular sugar were released from fat body of either stage. These observations indicate significant differences in the relations between cellular and haemolymph trehalose in the two stages.

TABLE III

Effect of bovine serum albumin (BSA) in preventing release of trehalose from cecropia pupal fat body during incubation in RW medium. Fat body from diapausing pupae (Batch II of Table II) was divided into 3–5 portions per pupa, which were analysed for sugar immediately or after incubation individually for 10 minutes in 5 ml of RW medium containing 0–5% BSA. Sugar concentrations are expressed in molalities as glucose equivalents (mean + S.E.M.)

	Concentration of BSA (%)	No. of Samples	Intracellular sugar (millimolal)	Retention, incubated/unincubated (%)
Unincubated tissue	—	7	38.0 ± 2.8	—
Incubated tissue	0	7	19.0 ± 0.8	50 ± 1
	0.5	3	18.9 ± 1.2	50 ± 2
	1.0	3	32.6 ± 2.8	86 ± 7
	2.0	4	33.9 ± 3.8	89 ± 9
	5.0	3	39.6 ± 0.7	104 ± 1

Effects of temperature on trehalose release from larval and pupal fat body

Since responses to changes in temperature should assist in distinguishing between downhill diffusion and energy-dependent transport as mechanisms for sugar efflux from cells, we examined the release of trehalose from larval and pupal fat body during incubation at different temperatures. In one series of experiments, the progressive appearance of trehalose from fat body in medium at 1°, 25° and 37° was monitored during 10 minutes of incubation (Fig. 3). From pupal tissue, significant trehalose was released at all three temperatures. Although the

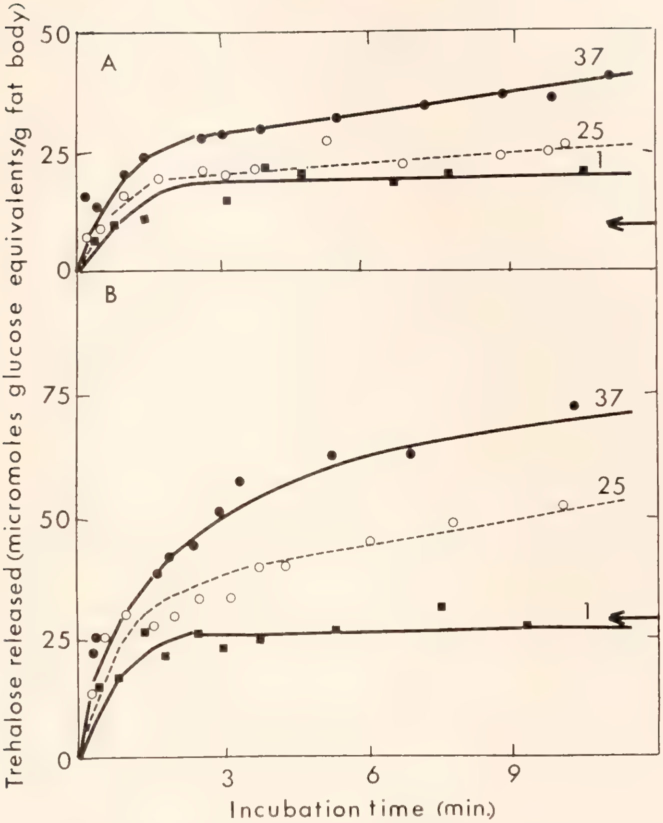

FIGURE 3. Effects of temperature on trehalose release from larval and pupal fat body. Pre-chilled fat body pooled either from (A) diapausing pupae (batch III of Table II) or from (B) feeding fifth instar larvae was divided into three 0.6 g portions, which were incubated in 4 ml of RW medium at 1°, 25° and 37° C, respectively. Samples (5 μl) of the incubation media were taken for analysis approximately every minute for 10 minutes. Arrows represent the amount of trehalose contributed by haemolymph adhering to the tissue, estimated from the average concentration of trehalose in the haemolymph of the animals and assuming 30% of haemolymph in the blotted tissue samples. Numbers are the temperatures of incubation.

data do not permit calculation of initial rates, it does appear that rates of trehalose efflux were less affected by changes in temperature than would be predicted if they were dependent on an enzyme-catalyzed, energy-dependent process. Our results are consistent with transport by passive diffusion. Larval fat body also released sugar at 25° and 37°, with release at 37 being augmented by net synthesis, as was shown when incubation was prolonged (data not shown). Unexpectedly, when larval tissue was incubated at 1°, the trehalose that appeared in the medium could be fully accounted for by adhering haemolymph. Thus, lowered temperature appeared to prevent sugar efflux from larval, but not from pupal fat body.

In a second series of experiments, the retained trehalose was measured after incubation at five different temperatures for one hour, a period chosen to ensure completion of the initial rapid phase of release (Table IV). Pupal fat body retained an average of 52% of its sugar content, with no significant influence of temperature. Larval tissue, on the other hand, retained only 20% of its sugar at 37° and nearly 80% at 1°, with values for trehalose retained at intermediate temperatures falling between these extremes. The ratios of the quantities of trehalose retained in larval fat body to the quantities retained in pupal fat body decreased with increasing temperature of incubation. The differential effects of incubation temperature on larval and pupal fat body were thus confirmed, the retention of sugar by larval tissue at 1° being particularly notable.

Pharate adults and "transitional" pupae. In early pharate adults at the second or third day of post-pupal development, haemolymph trehalose had risen to 30 mM, well above the level characteristic of pupae (Table I). The level in unrinsed fat body was approximately equal to that in the haemolymph. Also, about half of the intracellular sugar escaped during 10 minutes in RW medium, whether or not 2% BSA was included. Thus, the relations between fat body and haemolymph sugar in pharate adults resembled those in larvae rather than in diapausing pupae.

TABLE IV

Release of trehalose from larval and pupal cecropia silkmoth fat body incubated in RW medium at different temperatures. Larvae reared on foliage and 3 month old unchilled laboratory reared diapausing pupae were acclimated at 27° C. Fat body (0.2–0.4 g) was incubated in 2.0 ml of RW medium for one hour, blotted thoroughly, and intracellular sugar was determined (see Materials and Methods). Results are given in molalities as glucose equivalents, means ± S.E.M., with numbers of determinations in parentheses. Initial concentrations of trehalose in fat body can be calculated by dividing the concentrations remaining by the decimal fractions remaining. Initial concentrations varied somewhat because the different groups of animals were taken on different days. Haemolymph levels were about 40 mM glucose equivalents for pupae and 100 mM for larvae.

Temperature (°C)	Sugar remaining in fat body after incubation			Retention, remaining sugar/initial sugar (%)	
	Larval tissue (millimolal)	Pupal tissue (millimolal)	Larval/pupal (ratio)	Larval tissue	Pupal tissue
1	57.9 ± 7.3 (3)	19.6 ± 1.0 (4)	3.0	78.7 (3)	55.7 (4)
14	24.0 ± 2.3 (4)	11.8 ± 1.3 (5)	2.0	26.8 (4)	50.4 (5)
25	37.5 ± 2.7 (5)	20.4 ± 2.9 (6)	1.8	67.3 (5)	48.9 (6)
31	17.5 ± 2.3 (4)	11.7 ± 1.3 (5)	1.5	19.3 (4)	52.5 (5)
37	19.1 ± 2.6 (5)	18.0 ± 0.6 (4)	0.8	19.8 (5)	51.1 (4)

Pupae which have been chilled at 6° C for several months are no longer in diapause and have undergone physiological changes preparatory for development, although morphological development characteristic of pharate adults has not yet commenced (Shappirio and Williams, 1957). We termed such animals "transitional pupae." In our experiments, transitional pupae were characterized by fragile fat body tissue, which tended to dissociate into small fragments, and by elevated haemolymph trehalose (above 15 mM) (Table II, group G). We also found that certain groups of pupae which had been stored for several months at 25° C exhibited the characteristics of transitional pupae, even though they had not been chilled and presumably had not undergone endocrine activation for development (Williams, 1946); this even included some debrained pupae (Table II, groups E and F). Although their rates of respiration were below 20 μl/g-hr, all these pupae had haemolymph trehalose at levels well above those characteristic of typical early diapause, and nearly equal to their fat body trehalose. Their fat body also showed diminished capacity to retain intracellular sugar when placed in RW medium plus protein. Thus, in these pupae also, elevated haemolymph sugar was associated with enhanced tendency of the fat body cells to release trehalose.

The transition in the properties of fat body during storage of pupae is also illustrated in experiments on restoration of intracellular sugar after depletion in protein-free medium (Fig. 4). Fat body from pupae stored for 3 months at 25°

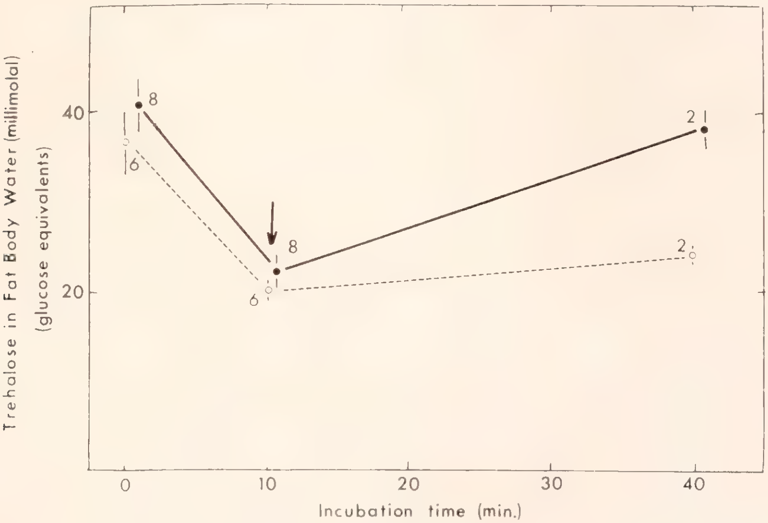

FIGURE 4. The effect of protein in restoring trehalose retentiveness in pupal fat body. Experimental procedure was as in Tables I and II, except that after the tissue was incubated for 10 minutes in protein-free RW medium (arrow), BSA was added to a concentration of 2% and incubation was continued for 30 minutes more; ●, diapause pupae stored 3 months (*cf.* column D of Table II); ○, transitional pupae stored 4.5 months (*cf.* column E of Table II). Vertical limits are standard errors of the means, and numbers are numbers of animals used.

was incubated for 10 minutes in RW medium to allow release of trehalose, whereupon 2% BSA was added. Sugar retentiveness was reestablished and (as a consequence of endogenous trehalose synthesis) its concentration was nearly restored. In a second comparable experiment using pupae that had been stored longer, restoration of intracellular trehalose did not occur, presumably because the cells remained non-retentive upon addition of BSA.

Injured pupae. Another physiological condition of interest is that caused by injury to the integument, a stimulus which, in diapausing pupae, is associated with activation of phosphorylase and breakdown of glycogen in the fat body (Stevenson and Wyatt, 1964), and results in temporarily elevated levels of haemolymph trehalose (Wyatt, 1961, 1963). In an experiment on this phenomenon, six days after injury, trehalose in both fat body and haemolymph were well above their pre-injury levels (Table II, group H). In addition, tissue placed in medium containing protein retained only 52% of its sugar, which was no more than in medium without protein, and less than that retained by tissue from uninjured diapausing pupae. Hence, the retentiveness of the fat body appeared to be decreased following injury to pupae.

Uptake of trehalose and other solutes into fat body

The experiments on release of trehalose by fat body *in vitro* indicated significant changes during ontogeny of the cecropia silkmoth, which suggested that changes in solute uptake might also be found. Penetration into fat body can be measured readily by observing the disappearance of a radioactive solute from

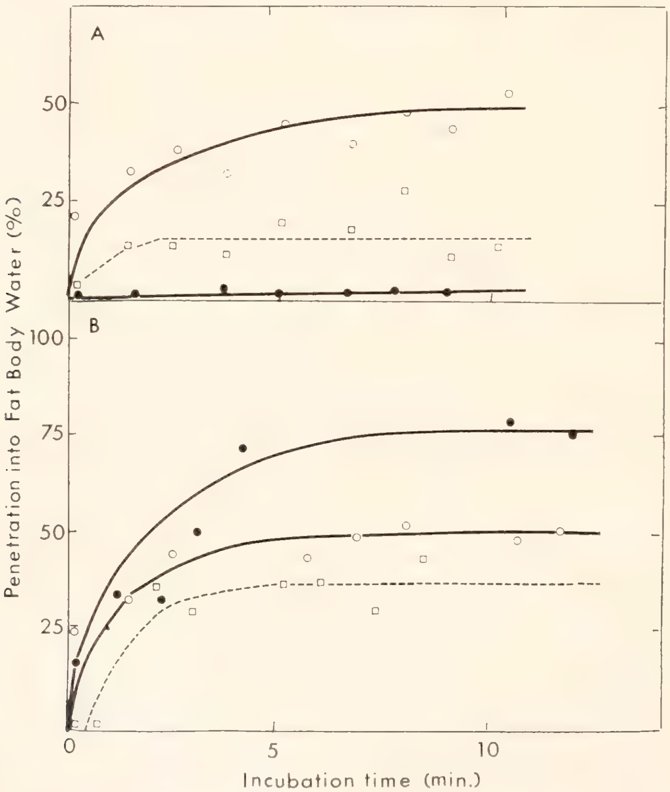

FIGURE 5. Representative curves showing solute penetration into fat body of cecropia silkmoth larvae and pupae. Fat body was incubated with ^{14}C-solutes together with ^{3}H-inulin, and percentage penetration into intracellular water was calculated as described in Materials and Methods. (A) Fat body from debrained diapausing pupae. (B) Fat body from fifth stage larvae. Tissue for glycerol and glucose uptake came from larvae 1 and 3 days after spinning; tissue for trehalose uptake was from fully-grown feeding larvae; ●, trehalose; □, glucose; ○, glycerol.

the medium in the presence of a second non-penetrating solute (such as inulin) with a different radioactive label, to serve as a marker for extracellular fluid. If the solute being studied is neither bound nor metabolized appreciably during the course of the experiment, then a measure of its distribution in the intracellular space is obtained.

Penetrability of cecropia fat body. Most experiments were conducted with ^{14}C-trehalose, ^{14}C-glucose or ^{14}C-glycerol, together with ^{3}H-inulin, as described in Materials and Methods. Typical patterns of uptake into larval and pupal fat body during 10 minutes of incubation are shown in Figure 5. Initial uptake was rapid, but the curves flattened off within 10 minutes at levels well below complete distribution in cell water. This supports the trehalose release data in indicating the existence of more than one compartment within the tissue. The extent of distribution of trehalose in larval tissue and of all three solutes in pupal tissue did not increase significantly when incubation was extended to one hour; with larval tissue, glucose and glycerol were metabolized during prolonged incubation. That the trehalose which penetrated intracellular water of larval fat body was not bound was shown by rinsing the tissue in K^{+}-Mg^{++} Ringer after incubation with ^{14}C-trehalose and measuring the radioactivity remaining. After two brief rinses, less than 4% of the trehalose that had penetrated the cells was retained.

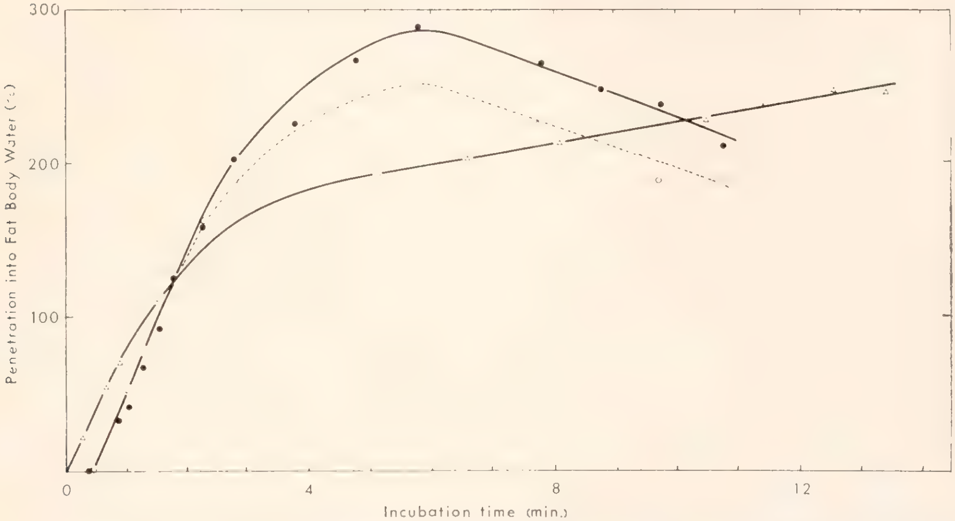

FIGURE 6. Uptake of ^{14}C-glucose *in vitro* into fat body from feeding fifth instar cecropia larvae in the presence or absence of unlabelled glucose or dinitrophenol. Uptake was measured in experiments similar to those of Figure 5. ○, ^{14}C-glucose, undiluted; ●, ^{14}C-glucose plus 50 mM unlabelled glucose. The two foregoing sets of points were obtained with the improved incubation vessel described in Materials and Methods. △, ^{14}C-glucose plus 1.6 mM 2,4-dinitrophenol, preceded by 3 hours of 6×10^{-5} M dinitrophenol *in vivo* (*cf.* Table VI).

Evidence on the mechanism of sugar uptake was sought by testing whether the rate of penetration of ^{14}C-glucose was affected by dilution with unlabelled glucose. Glucose, rather than trehalose, was used in this experiment because of its very low

TABLE V

Penetration of trehalose into cecropia fat body in different media. Penetration of ^{14}C-trehalose was measured in experiments similar to those described in Figure 5. Feeding, fifth instar larvae and pupae chilled 5 months were used

Experiment	Source of fat body	Medium and additions	Penetration of trehalose (%)
I	Larvae	RW medium	89
		RW + 50 mM trehalose	81
II	Larvae	RW medium	58
		Pupal haemolymph	80
	Pupae	RW medium	1
		Pupal haemolymph	3

endogenous level (less than 1 mM). Fat body from feeding fifth instar larvae (*i.e.*, before spinning) was used, in which uptake of glucose should be facilitated by its rapid conversion to trehalose. The uptake of ^{14}C-glucose *in vitro* was unaffected by the presence of 50 mM unlabelled glucose, which indicates that the process was not limited by a saturable carrier (Fig. 6). Because of conversion to trehalose, the apparent distribution of glucose in cell water greatly exceeded 100%, and the decline in this value after six minutes of incubation is probably explained by release into the medium of newly-synthesized ^{14}C-trehalose. In the experiments on uptake of ^{14}C-trehalose, we did not attempt to measure initial rates, but the presence of unlabelled trehalose did not alter the 10-minute penetration value (Table V). In the experiments presented in Figure 7 and Tables V–VIII, the medium generally included 50 mM unlabelled solute, and no difference was observed by its presence or absence.

To test whether metabolic energy might be required for maintenance of the fat body's stage-specific characteristics with respect to solute penetration, insects were injected with 2,4-dinitrophenol at doses sufficient to uncouple oxidative phosphorylation (Kurland and Schneiderman, 1959). Time was allowed for dis-

TABLE VI

Penetration of glycerol, glucose and trehalose into fat body of cecropia larvae and pupae treated with 2,4-dinitrophenol. 2,4-Dinitrophenol was injected 1.5 hour before dissection of feeding fifth instar larvae, or 3 hours before dissection of debrained, diapausing pupae, in amounts, estimated to give the stated concentrations when distributed in the haemolymph. In the experiment with larval tissue, dinitrophenol was also included in the incubation medium. Penetration of solutes into fat body in vitro *was measured in experiments similar to those in Figure 5, each using tissue from 3–5 animals*

Source of tissue	Dinitrophenol treatment	Penetration into fat body		
		Glycerol (%)	Glucose (%)	Trehalose (%)
Larvae	6×10^{-5} M *in vivo*, then 1.6×10^{-3} M *in vitro*	86	244	93
Pupae	4×10^{-5} M *in vivo*	57	38	19
	2×10^{-4} M *in vivo*	55	32	6

tribution of the drug, and its effectiveness was observed from the flaccidity of the animals. Fat body was collected and penetration of labelled solutes was measured. The initial rate of glucose uptake into larval fat body treated with dinitrophenol was no different from that in its absence (Fig. 6). In this experiment, the continuing uptake of ^{14}C-glucose after five minutes of incubation may be explained by reduction in trehalose synthesis and stimulation of glycolysis as consequences of uncoupling. From the data in Table VI, it is evident that after dinitrophenol treatment larval fat body took up trehalose freely and pupal fat body did not, just as in its absence (*cf.* Table VII). The penetrations of glucose and glycerol also did not differ significantly from those in untreated larval and pupal tissue, respectively (*cf.* Fig. 7). Thus, neither uptake nor exclusion of sugars or glycerol is dependent upon an unimpaired supply of ATP.

The rapid penetration of trehalose into larval fat body is in marked contrast with the general exclusion of disaccharides by cells of vertebrates. To test whether another disaccharide might also penetrate insect fat body, we used ^{14}C-sucrose. With larval tissue, sucrose distributed in 10 minutes into an apparent 124% of the intracellular water, showing that it penetrated rapidly and was metabolized to a small extent (Table VII). Pupal fat body, on the other hand, excluded sucrose to the same extent as it did trehalose.

TABLE VII

Penetration of disaccharides into fat body. Penetration of ^{14}C-trehalose and ^{14}C-sucrose was measured in experiments similar to those described in Figure 5. Feeding fifth instar larvae and debrained diapausing pupae were used

Source of tissue	Penetration into fat body	
	Trehalose (%)	Sucrose (%)
Larvae	85	124
Pupae (expt. 1)	11	12
Pupae (expt. 2)	12	10

Changes during development. During development of the cecropia silkmoth, no systematic changes were observed in penetration of the fat body by glycerol or glucose, though glycerol generally entered at a faster rate and to a greater extent than did glucose (Figs. 5, 7). Penetration of trehalose, on the other hand, underwent rather striking developmental changes. In fat body from feeding fifth instar larvae, trehalose distributed through 70–90% of the cell water. After the cessation of feeding, penetration of trehalose declined steadily during days 1–5 of prepupal development, approaching zero on the sixth day. During the 48 hours preceding ecdysis, trehalose penetration was restored to 45%, but then it declined in the fresh pupa to remain close to zero throughout diapause. Upon termination of diapause, penetrability to trehalose was restored. In fat body from animals at day 2 of pharate adult development (following activation by chilling) penetration of trehalose had risen to 60%, nearly the level observed with tissue from feeding mature fifth instar larvae. Later stages in pharate adult development could not be examined because the fat body becomes extremely fragile and cannot be handled.

To get an estimate of how quickly the fat body changes at the termination of diapause, development was initiated in brainless pupae by injection of β-ecdysone. Penetration of trehalose into fat body was still low after 18 hours, but by 42 hours after administration of the hormone it had risen to 53%, a value close to that found for day 2 pharate adults (Table VIII). As in natural development, uptake of glucose and glycerol remained unaltered during the response to ecdysone.

Haemolymph trehalose levels, measured at different stages of development, are also shown in Figure 7, and are consistent with developmental changes reported previously (Bade and Wyatt, 1962; Wyatt, 1967). The correlation of haemolymph trehalose level with penetrability of fat body by trehalose is notable. This sug-

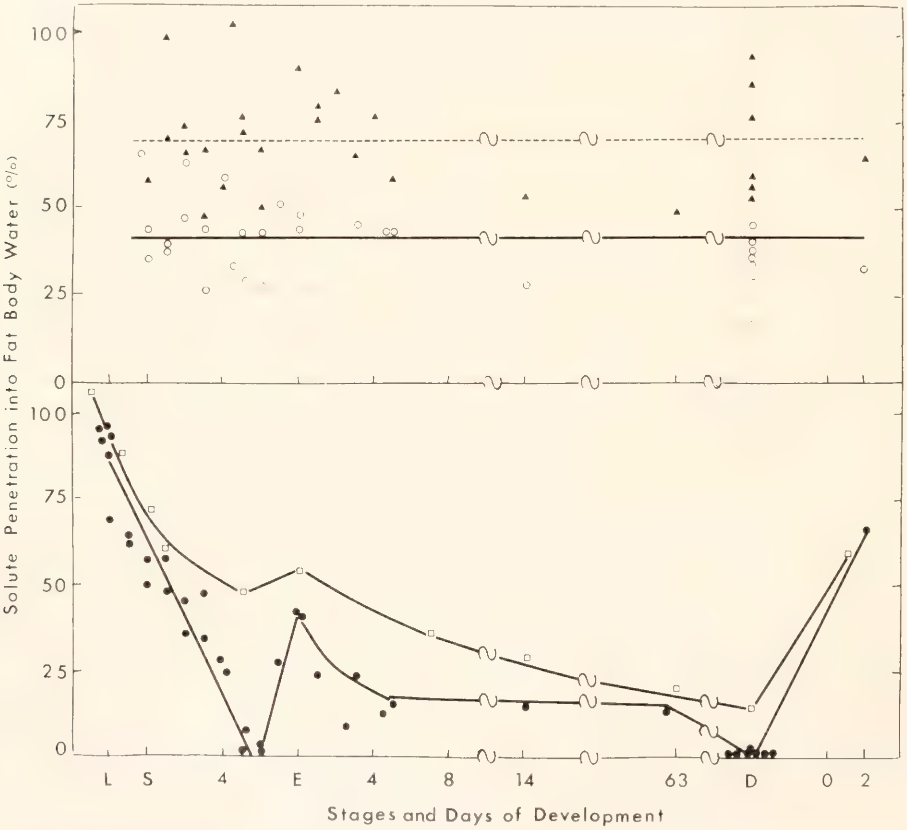

FIGURE 7. Developmental changes in the penetration of solutes into cecropia fat body and in the level of trehalose in the haemolymph; ▲, penetration of glycerol; ○, penetration of glucose; ●, penetration of trehalose; each point is obtained from an experiment similar to those shown in Figure 5; □, level of trehalose in haemolymph (mM, in glucose equivalents) estimated with anthrone in haemolymph pooled from animals at each stage: L, feeding fifth instar larvae; S, day of spinning; E, day of larval-pupal ecdysis; D, diapause. The stage following D is the early pharate adult.

TABLE VIII

Penetration of glycerol, glucose and trehalose into fat body of cecropia pupae after ecdysone injection or injury. Debrained diapausing pupae were selected for respiration less than 20 μl of O_2 per gram-hour. β-Ecdysone was injected at 2.5 $\mu g/g$ pupa weight. Experimental injury was performed as described in Materials and Methods. Penetration of ^{14}C-solutes was measured in experiments similar to those in Figure 5, each using tissue from 3–5 animals. Sugar levels were estimated with anthrone on pooled haemolymph samples, and are expressed as glucose equivalents

Experiment	Treatment	Penetration into fat body			Sugar level in haemolymph (mM)
		Glycerol (%)	Glucose (%)	Trehalose (%)	
I	β-Ecdysone				
	18 hours	56	40	3	24.3
	42 hours	63	41	53	34.2
II	Untreated control	85	42	0	20.2
	Injured				
	2 days	93	44	4	29.2
	5 days	75	16	10	37.6
	9 days	54	31	6	35.6
	20 days				31.4

gests that low blood trehalose is associated with the existence of a barrier between the intracellular water and the haemolymph.

Effects of injury. To test whether the response to injury (see above) was accompanied by altered fat body cell penetrability, eighteen debrained diapausing cecropia pupae were injured and, 2, 5 and 9 days later, fat body was collected and tested *in vitro.* Although the blood sugar level had almost doubled by the fifth day, penetration of trehalose into fat body was found to remain low and unchanged (Table VIII).

Penetrability of fat body in RW medium and in haemolymph. In the experiments that have been described, solute penetration into fat body was measured in RW medium. In case the tissue might behave differently in haemolymph, larval and pupal tissue were assayed for trehalose uptake during incubation in RW medium and in pupal haemolymph (Table V). Larval haemolymph could not be used without dilution because of its rapid clotting. In both media, larval fat body took up trehalose and pupal tissue did not. Therefore, we believe that the uptake of trehalose into fat body in RW medium is a valid indication of the properties of the tissue in its natural milieu of haemolymph.

DISCUSSION

Information on intracellular concentrations of metabolites is essential for understanding the operation *in vivo* of the mechanisms whereby metabolism is regulated. In insects, it is of especial importance to have such data for the fat body, since this tissue, as the chief center for intermediary metabolism and the synthesis of the organic constituents of the haemolymph, must play a key role in homeostasis. Our present data are apparently the first on the sugar content of insect fat body analyzed without prior rinsing, and they show that even quick rinsing of this tissue in osmotically equivalent salt solutions can lead to loss of 40–60% of the intracellular sugar. Previous data on the sugar content of rinsed fat body (see Introduction) are therefore subject to question. We do not know to what extent a similar caveat applies to other metabolites, but it is unlikely that charged molecules would pass through the cell membrane as readily as sugars.

The present analyses on unrinsed tissue, corrected for adhering haemolymph, indicate sugar (chiefly trehalose) in fat body water at levels that range from somewhat less than to more than double those in the surrounding haemolymph. Sugar levels in fat body below those in haemolymph raise a question concerning how the cells can supply sugar to the blood without active transport, a process for which no evidence exists in this system. The calculated intracellular concentrations are subject to some error because of uncertainty in estimating both the water content of the fat body and the amount of adhering haemolymph, properties which must vary with species and stage of development. If the actual water content is less than that assumed (70% or 50% of cell weight according to stage and species), the true intracellular concentrations would be higher than those calculated. That the mean sugar concentration in fat body water may actually be lower than that in the blood, however, is possible in view of indications of intracellular compartmentation which will be discussed below. One compartment might contain sugar at a relatively low level, while the level in another was high enough to permit transfer to the haemolymph by diffusion.

Intracellular sugar levels substantially above those in the haemolymph were observed in adult *Blaberus* and in diapausing pupae of the cecropia silkmoth. In these stages, the ability of the tissue to supply trehalose to the haemolymph is apparent, but a question arises as to how higher-than-ambient sugar levels are maintained in the cells. Upon brief incubation in an isosmotic medium containing inorganic ions and amino acids in proportions approximating those in lepidopteran haemolymph, sugar was lost from cecropia pupal tissue to the same extent as it was from larval and pharate adult fat body. Tissue from pupae in diapause, however, was distinctive in that its sugar content was largely retained when protein was included in the medium. This presumably accounts for its ability to maintain intracellular sugar above the haemolymph level *in vivo*. In our experiments, 2% bovine serum albumin or polyvinylpyrrolidone could substitute for cecropia haemolymph protein. But in the absence of amino acids, in a simple inorganic Ringer solution designed for lepidopteran tissue, fat body failed to retain its sugar even when protein was present. Thus, some synergism is implied between a macromolecule (protein or PVP) and amino acids in "sealing" the fat body cell membranes. In fat body from non-diapause stages, protein had little effect in preventing loss of intracellular sugar. On the other hand, when escape of trehalose from fat body was measured at different temperatures, larval tissue retained its intracellular sugar at 1° C while pupal tissue did not. This may reflect an adaptive change in membrane function at pupation, for pupae normally encounter below-freezing temperatures in nature. Thus, these two properties of fat body, namely retention of trehalose in protein-containing medium and temperature dependence of trehalose release, both change in the transition from larva to pupa of the cecropia silkmoth.

The implied developmental changes in cecropia fat body were confirmed and more fully explored by measurements of the penetration of radioactive solutes. In contrast to penetrability of glucose or glycerol, which did not change significantly during ontogeny, trehalose penetrated freely during the larval and pharate adult stages but scarcely during the pupal diapause. Measurements at daily intervals during the prepupal period showed additional transient changes. Unlike *H. cecropia,* diapause or non-diapause *M. sexta* failed to exhibit such changes during ontogeny (data not shown). In contrast to the changes in sugar release, those in the capacity of fat body to take in trehalose were manifest in protein-free medium. This is difficult to understand, and appears to indicate some asymmetry in the control of membrane transport. The developmental changes in cecropia fat body function with respect to both efflux and uptake of trehalose presumably reflect alterations in membrane structure.

The mechanism of sugar uptake by cecropia fat body cells has not been studied in detail, but the available evidence does not support the existence either of active transport or of a substrate-specific, carrier-mediated process like that known in mammalian tissues, including adipose tissue (Wilbrandt and Rosenberg, 1961; Crofford and Renold, 1965). Uncoupling of oxidative phosphorylation with dinitrophenol had no effect on penetration. Uptake of ^{14}C-glucose or trehalose was not affected by the presence of a high level of the non-radioactive compound. Sucrose, like trehalose, readily entered larval, but not pupal, fat body. These observations are consistent with simple diffusion, and suggest that transport into and out of fat body may be restricted by sieving based on molecular size, the pupal tissue admitting monosaccharides but not disaccharides.

The experiments on sugar release and those on uptake by fat body both gave results indicative of some sort of compartmentation within this tissue. The initial rapid release (within 2 minutes) of 40–60% of the intracellular trehalose was followed by little further loss during 30 minutes of additional incubation, even though a concentration gradient from cell contents to medium was always maintained. In other preliminary experiments (data not given), fat body transferred to fresh medium after 1 hour of incubation in RW medium, released little additional sugar. In our experiments on uptake, initial rapid transfer was followed by relative stability. The extent of occupation of cell water during 10 minutes incubation averaged 65% for glycerol and 40% for glucose, but ranged from zero up to 95% for trehalose depending on the stage of development. By comparison, in rat heart muscle, it has been stated that only 75% of the intracellular water was available for sugar distribution (Morgan, Henderson, Regen and Park, 1961). While there is kinetic evidence for functional biochemical compartmentation within various cell types (Threlfall and Heath, 1968; Sols and Marco, 1970), it is difficult to conceive of appropriate physical compartmentation in fat cells to account for our observations. Possibly, the nucleus or other organelles might constitute a compartment which does not readily exchange its trehalose with that in the haemolymph. Alternatively, although the fat body appears histologically homogeneous, some of its cells might exchange sugars less readily than others. Further study of this phenomenon is indicated.

The level of trehalose in the haemolymph of an insect at any time is a function of the rates of synthesis and utilization. Periods during which metabolic activity is high should be associated with increased use of trehalose, which might be expected to lower the level in the haemolymph. In cecropia, metabolic rates can be inferred from measurements of oxygen consumption (Schneiderman and Williams, 1953). This is maximal in the mature larva, which is also characterized by high haemolymph trehalose, however, and minimal during the pupal diapause, when haemolymph trehalose is low. Hence, the haemolymph trehalose level must be regulated primarily by the rate of production, not that of utilization. The fat body is the chief site of trehalose synthesis, and the output from this tissue may be influenced by (a) the supply of precursors, (b) the activities of enzymes and (c) release from the cells to the haemolymph. Let us consider each of these interdependent factors in turn.

Trehalose may be synthesized from free glucose after phosphorylation by hexokinase, from glycogen via the action of phosphorylase, or from glucose phosphates produced by gluconeogenesis. The transient rise in blood non-reducing sugar after glucose was fed to silkworms (Kuwana, 1937) is an example of stimulation of trehalose synthesis from the first source. The rise in blood trehalose after injury to cecropia pupae must be due, at least in part, to phosphorolytic breakdown of glycogen (Stevenson and Wyatt, 1964). But the elevation of trehalose after either of these stimuli is followed by return to "normal" levels, suggesting the resumption of control by long-term homeostatic mechanisms. As a possible regulatory mechanism at the level of precursor supply, Friedman (1968, 1971) has pointed to the activation of *Phormia* glucose-6-phosphatase by trehalose, which might lower the availability of glucose-6-phosphate for trehalose synthesis.

The rate-limiting enzyme in trehalose synthesis in cecropia fat body is trehalose-6-phosphate synthetase, which is subject to inhibition by trehalose (Murphy and Wyatt, 1965). Such feedback inhibition is, in our view, the principal mechanism operating to stabilize the level of this dissacharide in the fat body (*cf.* Friedman, 1967). Trehalose levels within the cecropia fat body vary much less than those in the haemolymph, and are regularly about half as high in pupal as in larval tissue. The mechanism by which trehalose is maintained at a lower level in the pupal than in the larval fat body is unknown, but, in this connection, the possibility that altered intracellular Mg^{++} may modulate the activity of the synthetase *in vivo* is currently being explored (Jungreis and Wyatt, 1972).

Our findings on the changing penetrability of fat body to trehalose provide an explanation for the markedly lower haemolymph sugar in the cecropia pupa than in the larva. In the larva, where trehalose escapes freely from the cells, its level must build up throughout the blood space before its intracellular synthesis can be blocked by feedback. In the diapausing pupa, since trehalose is retained in the fat body, intracellular feedback occurs while the level in the haemolymph remains far below that in the tissue.

The observations on certain intermediate developmental stages strengthen the case for an important role of fat cell penetrability in regulating hemolymph trehalose. Just before the larval-pupal ecdysis, a transient rise in penetrability to trehalose is accompanied by a simultaneous rise in blood sugar. In early pharate adults (obtained either by incubation of chilled pupae or by injection of ecdysone) increased penetrability and elevated haemolymph sugar appeared simultaneously. Certain batches of pupae which had been stored for more than 4 months (although still in diapause by the criterion of respiration) showed elevated blood sugar correlated with decreased capacity of fat body to retain intracellular trehalose. Fat body from injured pupae showed both elevated internal sugar (presumably resulting from activation of phosphorylase) and a decrease in ability to retain it (even though the low penetrability to external trehalose was unaltered), the two effects presumably contributing to bring about the rise in haemolymph sugar that is observed after injury.

The properties of insect fat body with respect to transport of sugars and the regulation of haemolymph sugar thus merit further attention, being profoundly different from the analogous functions of the tissues of vertebrates.

This research was supported by grants from the Whitehall Foundation and the National Institutes of Health, U. S. Public Health Service (HD-00032 and HD-02176). A. M. Jungreis was supported by Training Grant HD-00032 from the Institute of Child Health and Human Development. We are grateful to Dr. M. L. Pan for stimulating discussion and for help with many of the experiments.

SUMMARY

1. The levels of sugar (chiefly trehalose) have been determined in haemolymph and in unrinsed fat body (corrected for adhering haemolymph) of developing stages of the silkmoth *Hyalophora cecropia*, as well as a few individuals of other insect species. In cecropia, the fat body trehalose level was relatively stable at about 45 millimolal in mature larvae and about 20 millimolal in pupae, while that in the haemolymph ranged from somewhat above the tissue level in larvae to less than half the tissue level in diapause pupae.

2. During brief incubation in culture medium, fat body released 40–60% of its trehalose content. In larval tissue only, this release was sensitive to temperature, being blocked at 1° C. In fat body from diapausing pupae only, it was blocked by the presence of 1% or more of haemolymph protein, bovine serum albumin or polyvinylpyrrolidone in a medium containing amino acids.

3. Penetration of ^{14}C-solutes into fat body cells was measured. Trehalose and sucrose penetrated readily into larval fat body but not into pupal fat body; penetrability was restored in the early pharate adult. Glucose and glycerol distributed in 40–65% of cell water at all stages of development. Uptake was unaffected by dinitrophenol or by dilution with unlabelled solute. The data suggest that the mechanism of transport is simple diffusion restricted by molecular size.

4. The partial release of internal sugar during incubation of fat body and the partial occupation of intracellular water by exogenous solutes indicate the existence of some form of compartmentation in the fat body.

5. The developmental changes in retentiveness and penetrability to trehalose in cecropia fat body may explain changes in the steady state level of haemolymph sugar. In the larva, feedback inhibition of intracellular trehalose synthesis does not take place until trehalose has built up throughout the haemolymph, whereas in the diapausing pupa, the fat body retains trehalose, and feedback can occur within the cells while haemolymph trehalose remains low.

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FOOD AND FEEDING OF *PAEDOCLIONE DOLIIFORMIS* DANFORTH, A NEOTENOUS GYMNOSOMATOUS PTEROPOD

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Paedoclione doliiformis, like other gymnosomes, is a shell-less, holoplanktonic, opisthobranch mollusc. The species is unusual, however, in retaining larval characteristics throughout sexual maturity.

Danforth (1907) originally described *P. doliiformis* from collections made in Casco Bay, Maine, in 1902. This gymonosome has never been reported since, either from the type locality or other areas. I first collected this species during July, 1968, in the Gulf of Maine, and it has been taken regularly since then in the Gulf and shelf waters of Nova Scotia. The collection of numerous specimens of *Paedoclione* over a four year period has afforded the opportunity to expand and correct Danforth's original description and to study the behavior and ecology of this gymnosome.

Materials and Methods

Paedoclione doliiformis was collected from St. Margaret's Bay, Nova Scotia, and the Gulf of Maine, using conventional plankton nets towed at slow speed. Plankton tows were diluted immediately after collection in buckets of fresh sea water. Juvenile and adult specimens of *P. doliiformis* collect near the water surface where they are conspicuous by size, color, shape and swimming behavior; specimens were removed by pipette.

Specimens of *Paedoclione* were maintained in the laboratory in small containers of sea water kept at various temperatures between 2° and 20° C. Feeding experiments were carried out by placing *Paedoclione* in small glass containers together with mixed plankton or with thecosomatous pteropods of the genus *Spiratella* (*Limacina*). Feeding behavior was observed under a dissecting microscope.

Specimens used for anatomical studies were narcotized with urethane, then fixed in Bouin's fluid. Whole mounts were prepared by dehydrating specimens in an alcohol series, then clearing them in clove or wintergreen oil, and mounting them directly in Permount. For comparative purposes, larvae of *Clione limacina* (Phipps) were prepared in the same way. Whole mounts proved much more instructive for studying anatomical details than dissections. In addition, paraffin serial sections of 5 μ thickness were stained with Ehrlich's hematoxylin and eosin.

Results

Sexually mature *Paedoclione doliiformis* (Fig. 1) retains the small size and three ciliary bands characteristic of gymnosome larvae; thus the species is con-

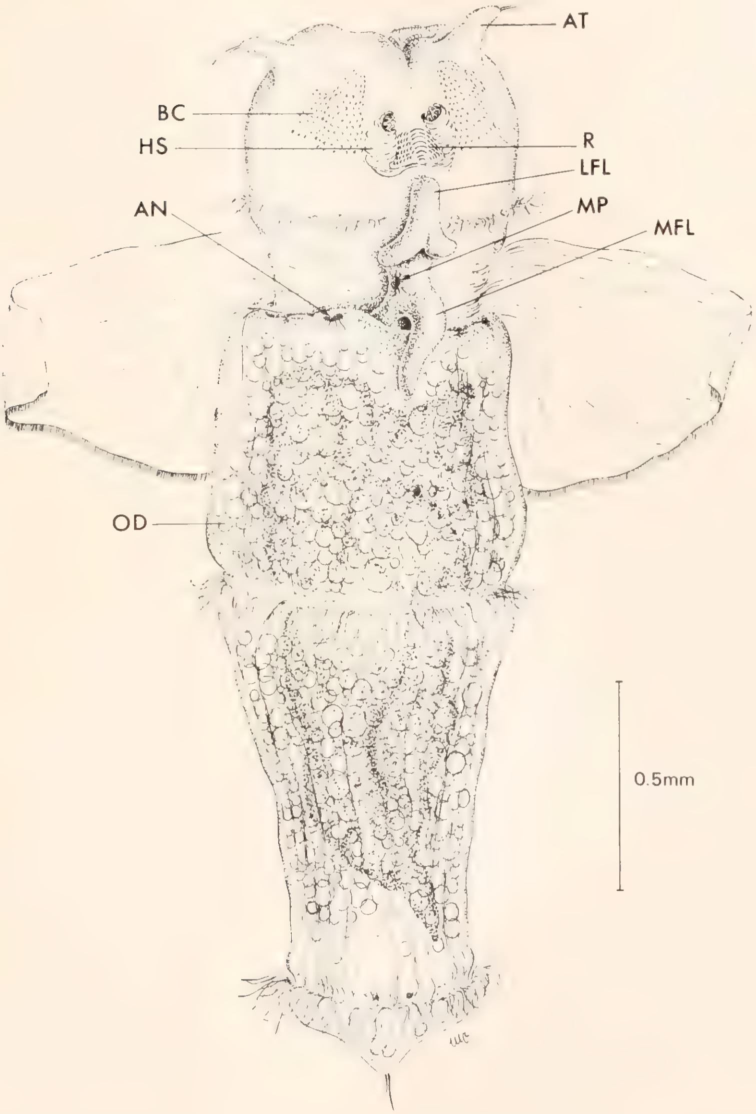

FIGURE 1. Ventral view of *Paedoclione doliiformis* with the retracted feeding structures as they appear in histologically cleared specimens; AN, anus; AT, anterior tentacle; BC, buccal cones; HS, hook sac; LFL, lateral foot lobes; MFL, median foot lobe; MP, male genital pore; OD, integumentary oil droplet; R, radula.

sidered to be neotenous. The maximum length of live, extended specimens is 2.5 mm. The three ciliary bands encircle the head, mid-body and posterior tip, with the discontinuous anterior band being interrupted ventrally by the foot. The distinct head bears a pair of retractile anterior tentacles. Very small posterior tentacles are located in depressions on the posterio-dorsal surface of the head.

The foot is divided into paired lateral lobes and a narrow, pointed, median lobe. The male genital pore opens between the right, lateral foot lobe and the median foot lobe, and anteriorly to the anus. The integument of the remainder of the body contains numerous oil droplets which obscure the viscera. There are no gills. The brown or brownish-orange viscera extend almost to the posterior tip of the body. The body is highly contractile, and the head, posterior tip of the body, and wings may be completely retracted so that the animal resembles a ciliated ball.

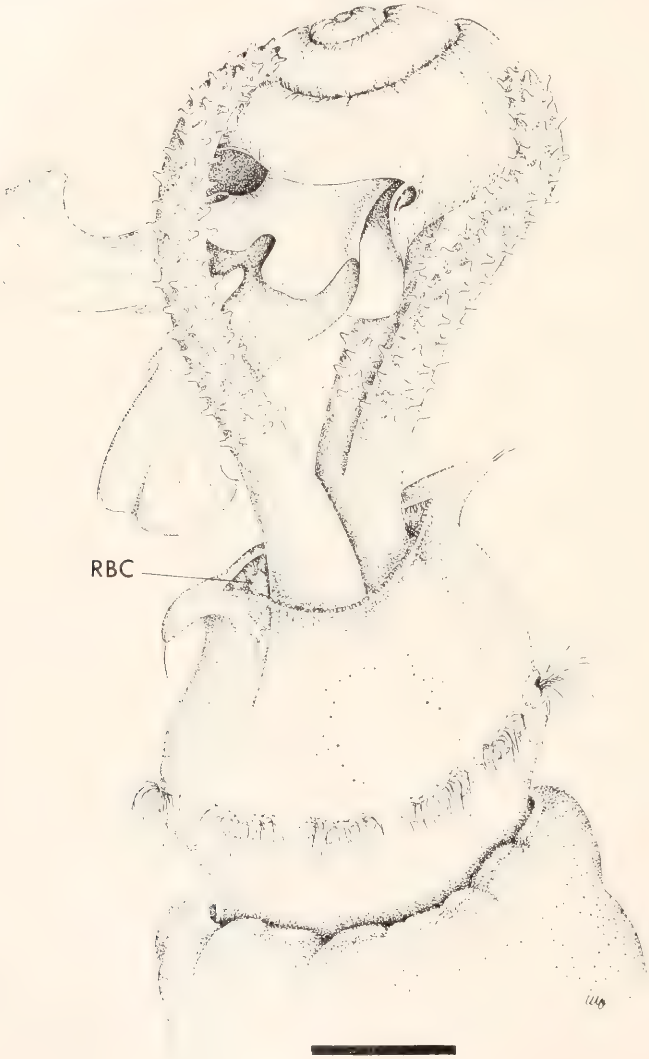

FIGURE 2. *Paedoclione doliiformis* capturing a swimming *Spiratella retroversa.*. Three buccal cones are everted to capture the prey; the papillae of the cones adhere to the prey's shell. The fourth, rudimentary buccal cone (RBC) remains retracted. Scale bar equals 0.25 mm.

All of the above features frequently are obscured in rapidly preserved, contracted specimens.

The wings are well developed, with cilia on their posterior edges. They move synchronously, dorsally and ventrally, during swimming. However, in the laboratory, the wings commonly are retracted completely, and locomotion is accomplished solely by movements of the cilia of the three bands.

Feeding observations

In the laboratory, *Paedoclione doliiformis* fed only on the thecosomatous pteropods *Spiratella retroversa* (Fleming) or *S. helicina* (Phipps). However, most feeding experiments were done with *S. retroversa* as it was the more commonly available species. *Paedoclione* showed feeding responses only to live prey, ignoring dead *Spiratella* or empty *Spiratella* shells. Other plankton offered as potential food, including prosobranch and bivalve veligers, never elicited a feeding response; nor were other recognizable food remains found in the gut of sectioned *Paedoclione*.

Paedoclione employs three, prehensile buccal cones to capture active *Spiratella retroversa* (Fig. 2). The buccal cones are everted and elongated immediately upon contact with the prey, and the buccal cone papillae adhere to and hold the shell of the prey during the initial stages of feeding. Active specimens of *Spiratella* usually struggle to escape by swimming, but are seldom successful. The buccal cones maintain their hold until the prey ceases violent movement or manages to retract into its shell, or until *Paedoclione* secures a grip with other feeding structures on the soft *Spiratella* body. The buccal cones then are withdrawn immediately to their resting position (Figs. 1 and 3) and play no further role.

Employment of the buccal cones is eliminated completely, however, if the prey is initially inactive or retracted into its shell. In these circumstances, the predator swims around the *Spiratella* shell seeking the aperture if the prey is retracted, or the base of the wings if it is extended and inactive. The proboscis is often everted at this time and its tip may move over the shell. The next stage of feeding proceeds only after the aperture is directly opposite the head of *Paedoclione*. If the *Spiratella* is retracted and the shell aperture sealed by the operculum, *Paedoclione* uses its proboscis or head as a wedge to force aside the operculum and gain access to the soft body parts of the prey.

From this point on, feeding on either extended or retracted *Spiratella* is completed in the same manner (Fig. 3). *Paedoclione* everts its proboscis and buccal mass, and the radular teeth and small hooks (Fig. 3, HK) are inserted into the *Spiratella* body. The teeth and hooks secure a grip on the prey and the proboscis alternately is pulled back and thrust forward. Each time the proboscis is pulled back, the radular teeth release, the radula is thrust further out of the proboscis, and the teeth secure a new hold further up on the *Spiratella* body. In this way, the entire *Spiratella* body is gradually pulled from its shell and ingested whole. Upon completion of feeding, *Paedoclione* leaves the intact, empty *Spiratella* shell.

Paedoclione generally eats small *S. retroversa* measuring less than 1.0 mm in diameter across the largest shell whorl. The total time occupied in the capture and ingestion of one *Spiratella* varied from 2 to 40 minutes and was dependent upon the initial activity of the prey and its size relative to that of the predator.

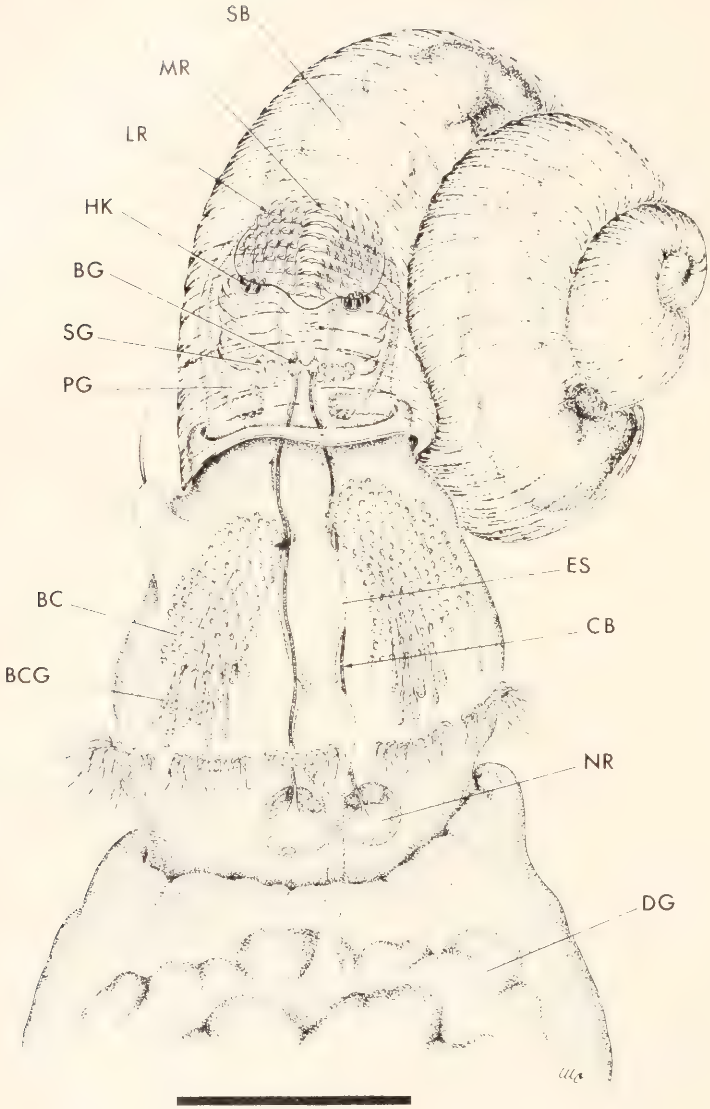

FIGURE 3. *Paedoclione doliiformis* extracting and ingesting *Spiratella retroversa.* The proboscis is everted into the shell aperature, and the radula is protruded from the mouth. The feeding organs within the proboscis are slightly enlarged to show structural detail. The buccal cones are retracted during this stage of feeding; BC, buccal cone; BCG, buccal cone gland; BG, buccal ganglion; CB, cerebrobuccal connective; DG, digestive gland; ES, anterior area of esophagus distended with ingested food; HK, hooks; LR, lateral radular teeth; MR, median radular teeth; NR, cerebral ganglion of nerve ring; PG, labial gland; SB, body of *Spiratella*; SG, salivary gland. Scale bar equals 0.25 mm.

Paedoclione occasionally was observed feeding on *Spiratella* larger than 1.0 mm diameter. *Paedoclione* began feeding on large, extended *Spiratella* by engulfing one wing at a time. The proboscis was placed against one wing at the base closest to the prey body. The radular teeth and hooks secured a grip on the wing tissue, and the wing gradually was engulfed by the alternate movements of proboscis and radula as described above. The predator began to ingest the remainder of the *Spiratella* body only after the second wing was engulfed in a similar manner. *Paedoclione* fed on both large and small retracted *Spiratella* by thrusting the proboscis into the shell aperture. However, if the shell is relatively large, *Paedoclione* may enter the aperture and first shell whorl farther than is possible with small prey. *Paedoclione* has been observed with the entire head, back to the wing level, thrust into a large shell to effect contact and extraction of the prey. *Paedoclione* feeding on large prey frequently stopped ingestion before complete extraction, leaving a portion of the prey body within the shell. Predation on large *Spiratella* presumably occurs only in the laboratory, and even then is infrequent. It seems unlikely for several reasons that *Paedoclione* could capture and ingest large *Spiratella* under natural conditions. Large specimens of *Spiratella* are faster swimmers than *Paedoclione*, the time necessary for extraction of relatively large prey is longer than for small, and, in nature, *Paedoclione* must support the weight of its prey once the *Spiratella* can no longer swim. Under laboratory conditions, feeding on large prey is accomplished on the bottom of feeding containers; in nature, the weight of a large *Spiratella* would cause the prey and predator to sink rapidly.

Specimens of *Paedoclione* also ate small *Spiratella helicina* on the few occasions when this species was available. The general feeding method is the same, but it is unlikely that *S. helicina* constitutes a regular food item of *Paedoclione*. This thecosome species occurs only rarely in the Gulf of Maine and Nova Scotian shelf waters, and *Paedoclione* is not known to inhabit other areas.

Spiratella retroversa shows various responses to contact with *Paedoclione*. It may react by vigorous wing movements in an attempt to swim away; this maneuver is successful only if the predator does not have a secure hold with the buccal cones or radula and hooks. *Spiratella* more frequently responds by retracting into its shell, closing the aperture with the operculum. The operculum does not appear to be an effective defensive structure as it can be easily pushed aside by *Paedoclione*. At certain times, however, the prey retracted so far into its shell that it was physically impossible for the predator to enter far enough to grasp the prey body. *Paedoclione* has been observed to spend as long as 20 minutes in unsuccessful attempts to reach retracted prey. It is also of interest that initially retracted prey never attempted to expand from the shell and swim once a *Paedoclione* began to enter the shell. The effectiveness of any prey defense observed in the laboratory should not be extended to natural conditions; the small volume of experimental containers probably conferred an unnatural advantage on *Paedoclione*.

Feeding apparatus

The feeding apparatus of *Paedoclione doliiformis* consists of the buccal cones, proboscis, paired hook sacs, radula and associated glands (Fig. 3). The buccal cones are retracted into the lateral walls of the head except during prey capture.

Contrary to Danforth's original description (1907), there are four, not three, buccal cones located dorso-laterally and ventro-laterally of the buccal cavity. The two cones on the right side are equally well developed. The left, dorso-lateral, buccal cone is the largest. The fourth cone, overlooked by Danforth, is rudimentary and is located ventro-laterally on the left side. The fourth cone usually is not obvious in dissections or cleared whole mounts; it can be demonstrated in histological cross sections.

The general structure of all four buccal cones is similar to that described for *Clione limacina* (Pelseneer, 1885; Lalli, 1970a), but histological details can not be made out clearly in paraffin sectioned material. The surface of each buccal cone is papillate, caused by clusters of projecting epithelial cells which are relatively longer than those of *C. limacina*. The papillae appear to be arranged in alternating transverse rows. Glands within the hemocoelic cavity of each buccal cone connect with the epithelial cells and presumably discharge their secretion to the surface of the cones. During feeding, three of the prehensile buccal cones are everted and elongated by increased blood pressure; the hemocoelic cavities within the cones are continuous with the cephalic hemocoel and there are no protractor muscles. Retraction to the resting position is accomplished by contraction of the several retractor muscles of each buccal cone. The fourth buccal cone only once has been everted; this occurred under unnatural circumstances when a specimen became entangled in fibers being used to slow locomotion for measurement (R. J. Conover, personal communication).

The anterior part of the gut of *Paedoclione* is everted during feeding to form a proboscis. Eversion is apparently accomplished by increased fluid pressure in this region as there are no protractor muscles. Changes in blood flow to the proboscis are apparently linked with flow to the buccal cones, as the proboscis and cones are never everted at the same time. The proboscis expands upon eversion and is capable of considerable elongation. The mouth is located terminally on the proboscis tip. Retraction of the proboscis is brought about by contraction of several retractor muscles which originate on the walls of the head and insert near the tip of the proboscis. Paired, multicellular, lobed glands open near the mouth (Fig. 3, PG); their function remains unknown.

The paired hook sacs and radula are connected and form the buccal mass proper. The hook sacs lie slightly dorsally of the radula and are connected with it laterally and posteriorly by muscle and connective tissue. The very small size and structural simplicity of the hook sacs was pointed out by Danforth (1907), who preferred to call them "ankistrophores." Nevertheless, these structures are homologous with the more complex hook sacs of other gymnosomes. Each sac is composed of intermixed muscle and connective tissue. It is noteworthy that the central areas of the sacs contain glandular cells with secertory material; this has not been reported in other gymnosome species. A maximum of seven, slightly curved, chitinous hooks permanently project from the anterior end of each sac into the buccal cavity. The hooks are similar to those of other species although they measure only 0.02 mm in maximum length. The hook sacs lack the complex musculature found in *Clione limacina* (Lalli, 1970a), but limited movement of the hooks apparently is possible during feeding. The hooks are inserted into the prey body during feeding and assist in ingestion.

The radular membrane, bearing the teeth, is supported by two lateral cartilages which form part of the odontophore. Figure 4 shows the shape of the radular teeth. The median teeth are sickle-shaped and lie in the groove between the odontophore cartilages. Completely formed mdian teeth have a small, single, median cusp. The five lateral teeth on each side of a median tooth have long spines and are exsertile. During feeding, the radula and hook sacs are forced out of the mouth and the radula protrudes beyond the level of the hooks (Fig. 3). The two odontophore cartilages rotate laterally, exposing the median teeth, and the lateral teeth erect as the radular membrane is stretched. The teeth are inserted into the prey tissue and the prey is pulled into the gut as the cartilages rotate medially and the radula is pulled posteriorly. Successive grasping actions of the radula continue until the entire prey body has been ingested. Although blood circulation to the radula has not been studied in detail, it is reasonable to assume that movements of the radula are controlled by changes in fluid pressure as well as by muscular action.

The hook sacs and radula are innervated by nerves from the buccal ganglia which lie dorsally of the esophagus and between the salivary glands. The buccal ganglia are joined with the cerebral ganglia of the nerve ring by long connectives.

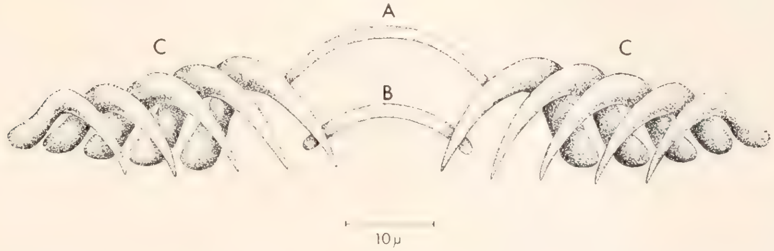

FIGURE 4. Radular teeth of *Paedoclione doliiformis*: A, fully formed median tooth with a median cusp; B, newly formed median tooth without a distinct cusp; C, lateral teeth.

Paired salivary glands are located immediately posterior to the hook sacs. The salivary ducts run anteriorly through the buccal mass and discharge separately into the buccal cavity.

The remainder of the digestive tract is structurally simple. The ciliated epithelium of the buccal cavity contains numerous unicellular glands. The extensible, ciliated espohagus passes through the nerve ring and opens directly into the lumen of the large digestive gland, where extracellular digestion takes place. The stomach is reduced to a small ciliated area on the right side of the digestive gland, as it is in other gymnosome species (see Morton, 1958; Lalli, 1970b). A narrow, ciliated intestine leads directly from the right side of the digestive gland to the anus.

DISCUSSION

Taxonomy

Paedoclione doliiformis has so far been reported only from the Gulf of Maine and Nova Scotian shelf waters. However, it seems unlikely that the species is endemic to these localities. Such a restricted distribution within areas where considerable water exchange takes place with the adjacent North Atlantic would be most unusual in a gymnosome species, or in other zooplankton groups. Since the species has not been familiar to many zooplanktologists, the possibility remains that *Paedoclione* has been overlooked or incorrectly identified in collections from other localities.

During the past four years, *P. doliiformis* has been a relatively common species in the collection areas. The absence of reported occurrences in the intervening 66 years between Danforth's collections and my own, in an area in which plankton collections have been numerous, has certainly been due to confusion with another gymnosome species. *P. doliiformis* frequently coexists with *Clione limacina*. Although the two gymnosomes are not anatomically similar, the differences are not always apparent between *Paedoclione* and the small polytrochous larvae of *Clione* which also have three ciliary bands. Live specimens usually can be distinguished by differences in coloration and general body form, *Paedoclione* having a darker brown or brownish-orange color and a truncated body shape compared with the conical form of *Clione* larvae. However, rapidly preserved, contracted specimens usually cannot be identified with certainty by cursory examination. The most rapid method of identification of such specimens is by the preparation and examination of cleared, whole mounts. The buccal organs stand out distinctly when these unstained preparations are examined under a compound microscope with the condensor lowered to increase contrast. The three largest, retracted, buccal cones of *Paedoclione* show clearly and extend from the anterior tip of the head to the level of the anterior ciliary band. The six, retracted, buccal cones of *Clione* larvae are shorter and concentrated only in the anterior part of the head. The hooks of *Paedoclione* number seven or less in each sac and are very short and blunt, while those of *Clione* are numerous, long and tapered, and extremely conspicuous as they extend throughout the length of the head. Radular teeth differences should not be relied upon as the preparations seldom separate the teeth sufficiently to determine structural details.

Two previously overlooked features of *Paedoclione doliiformis* are of considerable taxonomic importance. One is the rudimentary, fourth buccal cone. Following Danforth's description (1907), the major taxonomic character of *Paedoclione* has been the presumed unique asymmetry of the buccal cones. Thus *Paedoclione* was not considered to be closely allied with other gymnosome species which have one to three pairs of buccal cones, symmentrically arranged around the buccal cavity (Danforth, 1907; Tesch, 1950). Secondly, the presence of a cusp on the fully formed median radular teeth must be taken into account in taxonomic consideration.

There can be no doubt that *P. doliiformis* should be included in the family Clionidae. On the basis of similarity of buccal structure, *Paedoclione* is most closely related to the genus *Paraclione* (*Clionina*). Species of *Paraclione* also have two pairs of buccal cones, small hook sacs and unicuspid median radular teeth, but they differ from *Paedoclione* in other respects (Tesch, 1950; Pruvot-Fol, 1954). It is of interest to note that *Paraclione flavescens* (Gegenbaur) has been reported to have sexually mature larvae, although adults of 22 mm in length have also been found (Tesch, 1950).

Feeding

Paedoclione doliiformis feeds principally on *Spiratella retroversa,* although it is also capable of eating *S. helicina.* Food specialization on thecosmoes also has been reported for *Pneumodermopsis paucidens* which eats *Creseis* species (Sentz-Braconnot, 1965) and for *Clione limacina* which preys exclusively on *Spiratella retroversa* or *S. helicina* (Lalli, 1970a). Although the feeding structures differ among these gymnosomes, the general method of feeding is the same. All three gymnosome species capture thecosomes by prehensile structures, either buccal cones or sucker-bearing appendages, and extract the prey body from its shell by combined actions of the hooks, radula, and proboscis if present.

A comparison of feeding between *Paedoclione doliiformis* and *Clione limacina* reveals certain differences, although both eat the same prey species. *Paedoclione* preferentially feeds on veliger or small *Spiratlla retroversa* measuring 1.0 mm or less in shell diameter. Prey size preference of *Clione* changes with age; the youngest polytrochous larvae (0.3 mm in length) eat only veliger *Spiratella,* while the adults feed exclusively on large, metamorphosed prey (Conover and Lalli, in press). *Paedoclione* uses its three buccal cones to capture active *Spiratella,* but frequently eliminates this step of feeding if the prey is inactive or relatively small. Juvenile and adult *Clione* consistently use their six buccal cones to capture larger, metamorphosed *Spiratella.* Thus the number and relative importance of the buccal cones in feeding seems to be dependent upon the size and activity of the prey. In *Paedoclione,* the radula and proboscis are the most important organs used to extract the prey from its shell; the poorly developed hooks apparently play only a minor role. A true proboscis is lacking in *Clione,* although the anterior part of the gut can be everted to a limited extent; instead the well developed hooks are capable of extending far into the prey shell, and the hooks and radula are used for prey extraction and ingestion.

The frequent coexistence of *P. doliiformis* and *C. limacina* in the collection areas leads one to ask two basic questions. How can these two gymonosomes coexist when both feed exclusively on *Spiratella,* and what is the selective advantage of neoteny to *Paedoclione*? One possible explanation is that neoteny in one species limits food competition to the small-sized, more abundant prey. Competition for food thus would occur only between *Paedoclione* and larval *Clione,* with adult *Clione* feeding on the larger, less abundant *Spiratella.* My colleague, Dr. R. J. Conover, and I are presently attempting to examine this relationship between *Paedoclione* and *Clione* by laboratory studies on the size of prey eaten *versus* predator size, and by comparing feeding and growth rates of *Paedoclione* and *Clione* both in pure and mixed cultures. We are also attempting to gather information on the life cycles of both gymnosomes and of *Spiratella retroversa* in Nova Scotian shelf waters to determine abundance and size of the predators and their prey during times of coexistence.

I wish to gratefully acknowledge the assistance of Dr. Robert J. Conover with the research and preparation of this manuscript. I also wish to thank the Bedford Institute of Oceanography, Dartmouth, Nova Scotia, and the Fisheries Research Board Biological Station, St. Andrew's, New Brunswick, for permission to participate in several cruises. Final illustrations were prepared by Margot Oeltzschner. This research was supported by Grant #A5248 from the National Research Council of Canada.

SUMMARY

The neotenous gymnosome, *Paedoclione doliiformis,* feeds exclusively on the thecosomatous pteropods, *Spiratella retroversa* or *S. helicina.* Three prehensile buccal cones are used to capture prey; the fourth buccal cone is rudimentary. *Paedoclione* employs its proboscis, radula and hooks to completely extract the prey body from its shell.

Paedoclione doliiformis frequently coexists in the collection area with another gymnosome, *Clione limacina,* which is also a feeding specialist on *Spiratella. Paedoclione* and polytrochous larvae of *Clione* preferentially select small-sized prey of less than 1.0 mm shell diameter and thus are potential competitors for food; adult *Clione* feed exclusively on *Spiratella* larger than 1.0 mm. It is suggested that neoteny in *Paedoclione* permits coexistence of these two gymnosome species by limiting food competition to the small-sized, more abundant prey.

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THE PROMPT DETECTION OF IONIZING RADIATIONS BY CARPENTER ANTS[1]

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The ability of animals to detect ionizing radiations has been known almost since the discovery of x-rays, but only recently has it become widely appreciated. The prompt detection of ionizing radiation has now been thoroughly documented for several species of mammals and for some invertebrates (see reviews by Kimeldorf and Hunt, 1965; Smith, 1971).

Intensive behavioral and electrophysiological studies have revealed that detection is mediated primarily *via* visual and olfactory sensory systems in mammals. Little is known about the routes of detection available to lower organisms. Prompt behavioral reactions to x-rays by coelenterates (Kimeldorf and Fortner, 1971) indicates that a highly organized receptor system is not essential for detection. Periodic attempts to identify the mechanisms by which ionizing radiation leads to prompt, reflex-like responses of arthropods have implicated photoreceptors (Axenfeld, 1897; Baylor and Smith, 1958; Smith and Kimeldorf, 1964; Terwilliger and Levy, 1964). Other sensory systems have not been studied, yet arthropod antennae are laden with a variety of sensory receptors and, as distinct appendages, are well suited to manipulative procedures and electrical recording.

The primary objective of the present study was to seek out and characterize prompt behavioral responses given by insects to bursts of ionizing radiation, and to uncover the mechanisms through which detection occurs. In this manner, we hoped to extend the body of knowledge on a topic that has developed almost entirely from studies on selected vertebrate species. Carpenter ants were chosen as subjects for study because some species have been reported to respond immediately (within four seconds) to x-rays (Hug, 1960), yet nothing is known about how radiation effects these responses in ants.

METHODS

Behavioral studies

Major worker ants (10–15 mm long) of the species *Campanotus herculeanus* served as experimental subjects for this study. Ants were collected from nests within rotting logs near Corvallis, Oregon. The ants were maintained in the laboratory at room temperature and an approximate 12/12 photoperiod, for at least several days prior to experimentation. Water was provided with moistened cotton pads, and food consisted of bits of insect bodies and grains of sugar.

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[1] This investigation was supported by the General Research Fund, Oregon State University Graduate School, and by USPHS, Food and Drug Administration, Bureau of Radiological Health under research grant RL 00119. D. L. Martinsen was the recipient of a NSF Faculty Fellowship and a traineeship in environmental toxicology, USPHS grant ES 00055, during this study.

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Prompt, reflex-like responses of ants to x-rays are defined as reactions that were elicited within a few seconds of the onset of exposure and were transient, ceasing either during or soon after termination of exposure. Some consistent, readily-identifiable prompt responses included head bobbing, brisk waving of the antennae, and a startle response involving an abrupt, rapid running behavior. The antennal reaction and the running behavior were selected for quantitative study. Latency of response was defined as the time duration from the onset of exposure to the start of a specific response. Strength or intensity of the running response was assumed to be reflected by the speed of running and was determined by measuring the distance traveled per unit time (cm/sec or cm/min). Other characteristics of prompt responses that were expressed quantitatively were the per cent subjects responding, and the duration of a response.

X-ray stimuli were delivered with a General Electric Maxitron 300 therapy unit. The port of the x-ray unit was positioned above an ant exposure chamber, which in turn rested atop 15 cm of high-density rubber pads in order to maximize backscatter. Most exposures were made at 300 kVp and 20 mA, half value layer equivalence (HVL) = 1.8 mm aluminum (Al). Exposure rates below 0.7 R/sec were achieved by either increasing the beam filtration with thin absorbers, or decreasing the maximum kilovoltage applied to the tube. These variations in beam quality were employed only to determine the exposure rates below which no prompt responses could be elicited. To measure an exposure rate, a Victoreen thimble was inserted into the center of an ant test chamber and exposed under experimental conditions. The distance between the x-ray target and the chamber was then adjusted to yield the desired exposure rate. The test chambers for ants were designed to be small enough, relative to the exposure field, that the measured variation in rate throughout the chamber was usually not greater than the variation among three readings taken at the center of the chamber.

Ants were exposed in single-compartment, 30-cc plastic tissue culture flasks (2 cm × 3.5 cm × 8 cm). The chambers were perforated to provide ventilation and easy introduction of food and water. Experimental subjects were transferred from the main laboratory colony to the chambers between 24 and 30 hours prior to exposure.

Remote observation of ants during x-ray exposure was made by a closed-circuit television system. A television camera, equipped with an f 1.4, 25 mm lens, was positioned close to the exposure chamber in the x-ray room. A TV monitor and video-tape deck were on line and set up in the x-ray control room. An audiochannel of the tape deck allowed the recording of a tone emitted by the x-ray generator that corresponded to the precise period of exposure. Tapes were replayed in the laboratory for the extraction of data on the latency, strength, etc. of the behavioral response in question.

Preliminary tests revealed no difference in the responses between ants exposed individually and those exposed in small groups. Thus, unless stated otherwise, ants were maintained and subjected to test stimuli in groups of five to eight ants per chamber. To minimize the risk that spontaneous changes in behavior would be interpreted as radiation-induced, subjects were not exposed until they achieved and maintained a resting posture for at least one minute prior to exposure.

Several odors were used to test the behavioral responses of normal and experimental subjects to olfactory stimulation. The strongest and most predictable response (immediate attack behavior) was elicited by the natural odor of a foreign ant (an ant of a different species). This stimulus was presented by introducing a freshly-killed foreign ant, impaled on a toothpick, into the test subjects' chamber. The odors of nestmates were introduced as controls, to insure that visual or mechanical cues were not involved in the responses observed.

Both partial and complete antennectomy and ophthalmectomy were employed to assess the role of antennal and visual receptors in the detection of ionizing radiation. Operations were performed on ants anesthetized with carbon dioxide. Control subjects for each experiment underwent a sham operation procedure. After perfection of surgical technique, the mortality of experimental subjects was no greater than that of anesthetized controls (0–20%). In all cases, a post-operative recovery period of at least 24 hours was allowed prior to experimentation. One or both antennae (or parts thereof) were removed by cutting between two adjoining segments with irridectomy scissors. Care was taken to cut precisely at the intersegmental joint, thereby preventing excessive loss of body fluid, and facilitating quick recovery. To insulate the intact antenna from odors in one study, purified white shellac was applied to the antennal flagella with the aid of a fine-tipped brush. The shellac was nontoxic, dried quickly, and formed a seamless coat over the flagella. For ophthalmectomy, a fine watchmakers forcep was employed to remove the cuticle, corneal lens, and underlying crystalline structures containing the receptor cells. It was not entirely possible to be certain by visual inspection through a microscope that all photoreceptive cells in the compound eye had been removed or destroyed, but operated subjects did not, after dark adaptation, respond to bright flashes of light, as did sham-operated controls.

Electrophysiological studies

The Electroretinographic response (*ERG*). Preparation of the eye for ERG recording involved severing the head from the body, removing the antennae and mouthparts, then partially imbedding the isolated head in wax. The wax mount was held in a stereotaxic device. With the aid of micromanipulators and a binocular microscope, a platinum-clad microelectrode was inserted into the cuticle of the eye to a depth of about 0.2 mm, and a platinum reference electrode was positioned in the base of the head. All plastic components were painted flat black, to prevent any potential radiation-induced fluorescence from stimulating the eye.

Repeated tests showed that the responsiveness of the eye did not begin to decrease for at least three hours after initial records. All results were obtained from fresh preparations. The ERG signal was amplified and recorded on a Grass VII oscillograph in conjunction with a Grass 7PIA low-level DC preamplifier.

We exposed the eyes of ants to stimuli of light, beta radiation, and x-rays.

The typical procedure was as follows: Each eye was dark-adapted for several minutes, then stimulated by flashes of light for standardization of response, followed by test stimulation with ionizing radiations. The physiological integrity of the preparation was tested at intervals by checking the response to visible light stimuli. In this way, comparisons could be made between responses given to ionizing and non-ionizing radiation, using each eye as its own control.

For stimulation by light, a Bausch and Lomb diffraction grating monochromator in series with a 150-watt xenon lamp system was used to provide 500 nm wavelength radiation. The light beam was passed through the aperture of a light-tight box containing the eye preparation. Flash duration was controlled by a leaf-type camera shutter fixed to the exit slit of the monochromator. Bimetallic quartz-bonded interference filters of neutral density were used to change the dose rate over several log units. Intensities were measured in ergs/cm^2-sec at the level of the eye with a YSI-Kettering radiometer.

Beta radiations were provided by compact, sealed applicators (Tracerlab) containing either 100 mCi or 50 mCi of strontium-90 in equilibrium with yttrium-90. The maximum energy of beta radiation emitted by the source was 2.25 MeV. Dose rate measurements were made by means of lithium fluoride thermoluminescent dosimetry. Very small calibrated lithium fluoride dosimeters were positioned at the site of the eye, and the absorbed dose determined for timed exposures in the range of 1/20 to 1/60 seconds. For presentation of the eye to beta radiation, an apparatus described by Smith and Kimeldorf (1964) was employed. The outer margin of a steel disc was positioned between the beta source and the eye preparation. The disc had two holes drilled in its margin, 180 degrees apart, and was aligned so that rotation of the motor-driven disc exposed the eye preparation to two flashes of radiation per revolution. As one hole exposed the eye to beta radiation, the opposite hole exposed a photocell to a flash of light from a shielded light source. Output from this photocell led into one channel of the oscillograph and served as a stimulus monitor.

X-ray exposures were made with a diagnostic x-ray unit, operated at 70 kVp with tube currents between one and 10 mA, and a measured HVL of 0.58 mm Al. The x-ray tube head was mounted on top of a lead-lined cabinet, which served as the exposure chamber. X-ray exposures were controlled by a solenoid driven focal plane shutter constructed of lead. Limitations imposed by the relatively high-inertia shutter made it impossible to attain stimulus rates of greater than four per second or durations of less than $\frac{1}{10}$ second. The onset and duration of each x-ray exposure was monitored by a photocell that was covered with a fluorescent screen and enclosed in a light-proof capsule. The photocell was suspended in the exposure chamber directly beneath the eye preparation and its output was fed into one channel of the recording oscillograph. Exposure rates were varied by changing tube current. A low energy 25 R thimble chamber was positioned at the site where the eye was to be exposed and measurements were made at several tube current settings. The exposure rate over the range tested was linearly related to tube current and ranged from 0.3 to 2.7 R/sec.

The Electroantennographic response (EAG). The composite bioelectric response of the insect antenna, named the electroantennogram by Schneider (1957a), has been less well studied than the ERG. When an adequate olfactory stimulus impinges on the insect antenna, a relatively slow electrical potential difference develops between the tip and the base. This potential is thought to reflect the summed receptor potentials of many olfactory sensory neurons (Schneider, 1963).

The ant antenna was isolated from the rest of the body to record the EAG, because stimuli often evoked potentials associated with muscular movements of the mandibles or of the scapus muscles joining the antenna to the head. The isolated antenna was mounted on a cork platform. Two platinum electrodes were affixed to separate micromanipulators. The tip of one electrode was then inserted into the base of the antennal scape. The other electrode was secured within a fine-tipped glass pipette filled with 1.0% saline, into which the distal segment of the flagellum was manipulated. Amplification and recording was accomplished by the systems described for ERG measurements. All results were obtained within 30 minutes of isolating an antenna, after which dehydration usually resulted in diminished responsiveness.

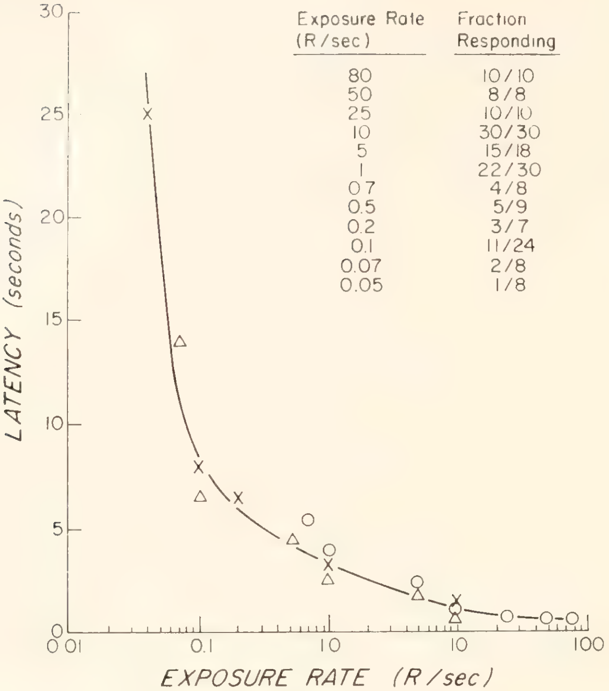
To administer odor stimuli, an air-jet system was devised which provided a constant flow of air over the isolated antenna, into which a test odor could be injected periodically. Odor stimuli were presented by dipping the tip of a clean glass rod into a test solution and passing it back and forth through the air stream impinging on the antenna. Control tests were made in the same way, using distilled water rather than an odorous solution. The approximate onset and duration of each stimulus was recorded on a separate channel of the oscillograph using a hand-operated switch.

The 50 mCi radioactive source was used to provide beta radiations for the antenna study. The air-jet was removed and a lead shield was placed over the antenna, with a small window located directly above and one centimeter out from the antenna. To deliver a train of stimuli, the beta source was passed back and forth over the window. The rate of movement of the source determined the stimulus duration, whereas intensity was varied by altering the distance between the source and the antenna. Dose rates from 0.15 to 3.2 rads/sec were available by this procedure. Each exposure to beta radiation was preceded and followed by a sham exposure with a Tracer-lab non-radioactive applicator. Sham exposures were made to detect any potential artifacts that could be induced by the manipulation of the radiation source close to the antenna.

Results

Prompt behavioral responses to x-rays

Intact (normal) subjects. Below an exposure rate of 0.05 R/sec, no ant gave any behavioral evidence of detection. Above this threshold rate, the proportion of subjects responding by vigorous movements of antennae was a direct function of exposure rate (see tabular data, Figure 1). This response occurred through a wide range of exposure rates and beam qualities. It appeared to represent a taxis or orientation response. Antennal waving was always the first response given to x-rays, and often was the only response at low exposure rates (less than 0.5 R/sec). The latency of the antennal-waving response was an inverse function of the exposure rate (Fig. 1). Note that the latency varied through three orders of magnitude. Lower exposure rates could be achieved only by using different beam qualities.



Exposure Rate (R/sec)	Fraction Responding
80	10/10
50	8/8
25	10/10
10	30/30
5	15/18
1	22/30
0.7	4/8
0.5	5/9
0.2	3/7
0.1	11/24
0.07	2/8
0.05	1/8

FIGURE 1. Average latency of the antennal-waving response (graph), and fraction responding (tabular data), as a function of exposure rate. Different symbols represent different beam qualities: 0 = 300 kV, 20 mA, 1.8 mm Al. HVL; X = 300 kV, 20 mA, 4.0 mm Cu. HVL; Δ = 100 kV, 20 mA, 0.25 mm Al. HVL.

Since there was no significant difference in the average latency of the response at points of common exposure rates (10, 1 and 0.1 R/sec), the curve in Figure 1 was fitted to an average value at each exposure rate where more than one beam quality was employed.

Another behavior that consistently appeared as a prompt response was a burst of rapid running activity along the floor and all sides of the exposure chamber. As with the antennal response, the incidence varied directly, while latency varied inversely, with exposure rate. The exposure rate threshold for the running response appeared higher than for the antennal response since it was not observed with less than 0.2 R/sec. Rates of 1 R/sec or less often evoked only the antennal response; when running did occur it was typically several seconds later in appearance than the antennal-waving response. At 10 R/sec and above, the running response began simultaneously with the antennal response, and both responses were exhibited by 100% of the subjects. The intensity of the running response appeared variable until it was analyzed as a function of exposure rate. When speed was taken as a measure of the strength of the running response, it was found to be related to the rate of x-ray exposure (Fig. 2). Speed of running appeared to be independent of exposure rate between 0.5 and 5 R/sec, then increased dramatically up to 80 R/sec.

Some ants exhibited adaptation during exposure at rates below 0.5 R/sec during prolonged exposures, *i.e.*, the antennal response waned, and subjects ceased to give behavioral evidence of detection while the beam was still on. Rates of 1 R/sec or higher, however, usually caused antennal waving and running to persist throughout a 30 second exposure period. During long (minute) exposures, ants would periodically stop running and clean their antennae, but these brief pauses were transient. Rapid running was quickly resumed, and there were no clear indications of adaptation or habituation at rates of 1 R/sec or higher.

On termination of exposure, most subjects exhibited an abrupt cessation of running, accompanied by vigorous antennal-cleaning behavior. Thus, both the onset and the cessation of irradiation elicited detectable changes in the behavior of ants. At rates of 10 R/sec or higher, the "off" response was transient, lasting only a few seconds. A high level of activity then reappeared, even though the beam had gone off.

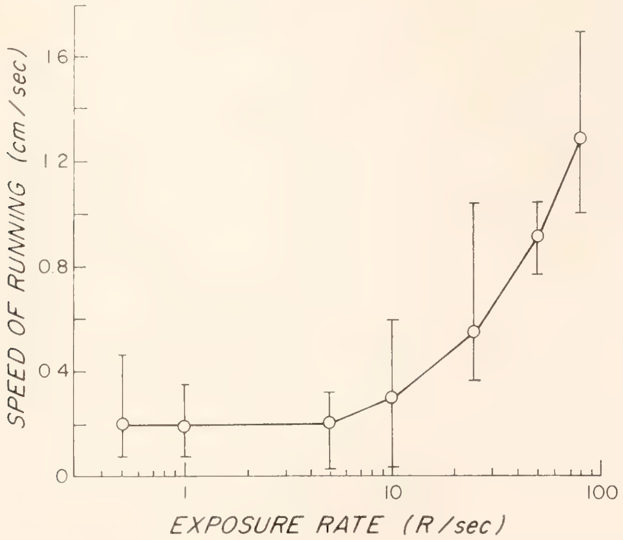


Figure 2. Average intensity of the running response as a function of exposure rate. Each point is an average of 5–8 subjects. Vertical bars show range of response.

A test was made to determine the effects of repetitive exposure on prompt responses. Alterations in the response pattern might be expected as a result of sensitization, fatigue, or direct tissue damage. A group of 10 ants was subjected to six exposures during a two-day period. Each exposure, at 80 R/sec, lasted for one minute. There was no measurable change in the latency or the strength of prompt responses with successive exposures.

Ophthalmectomized subjects. The prompt nature of the foregoing responses indicated that the nervous system was being stimulated, either directly or indirectly, possibly through specific sensory pathways. To test for potential involvement of the visual system, we bilaterally opthalmectomized two groups of ants (n = 5/

group) and exposed them to 10 R/sec for 30 sec. Responses were just as strong as those given by normal controls. The average latency of the running response was somewhat longer in eyeless subjects (1.2 sec) than in normal ants (0.9 sec), but this difference was not statistically significant. Thus, the visual system was not primarily responsible for the behavioral reactions given by ants to brief exposures of x-rays.

Antennectomized subjects. Several experiments were performed in an attempt to elucidate the role of the antennae in responses given by ants on exposure to x-rays. When stimulated at 10 R/sec or higher, antennal-waving and running behavior began simultaneously, usually within one second of the onset of exposure. To determine whether intact antenna were essential for the running response to be elicited, we removed both antennae from each of six subjects, then exposed them to 10 R/sec for 30 seconds. None of the antennectomized ants gave any detectable response during exposure. Antenna thus appeared essential for the detection of a stimulus which led to the running response given by normal subjects.

A subsequent experiment established that ants missing only the flagellar portion of both antennae were not capable of detection. In the type of antenna possessed by ants, aggregations of specialized sensory nerve endings are found only in the flagella. By contrast, antennal muscles and motor components of the large antennary nerves are restricted to the scapes (Schneider, 1964). The sensory component of the nerve tract extends from the deutocerebrum through the scapes into the flagella. Since ants with scapes intact did not respond to x-rays, ionizing radiation must act primarily or exclusively on the receptor neurons distributed along the segments of the antennal flagella.

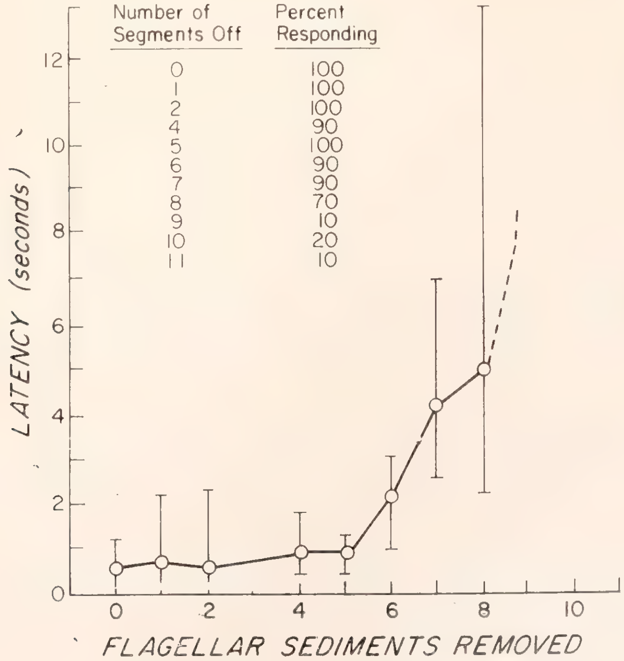
Thus, a study was made to determine which of the 11 flagellar segments were involved in detection. Eleven experimental ($n = 10$/group) and one control groups were prepared. Members of each experimental group had "n" segments removed from both antennal flagella. The "n" was equal to one segment for the first group, and was increased by one for each succeeding group, such that ants in group 11 had all eleven segments (the entire flagellum) missing from both antenna. Each ant was maintained individually and exposed to 10 R/sec for 30 seconds. Latency and strength of behavioral responses were scored, as well as the per cent responding to exposure.

The latency of the running response is plotted in Figure 3 as a function of the number of segments removed. Per cent subjects responding is also tabulated in this figure. Note that nearly all ants in each group missing fewer than six segments responded as quickly as did the controls. Most ants missing 6, 7, or 8 segments did respond, but the average latency of the response increased markedly. Almost none of the subjects missing nine or more segments gave evidence of detection during the exposure period (in other words, latency to response became infinite).

The strength of prompt responses (frequency of antennal-waving and speed of running) followed the same pattern as latency. Ants with more than six segments intact responded as strongly as did the controls. When the sixth, seventh, and especially the eighth segments were missing, subjects usually signalled detection only by mild waving of the antennal "stubs," and responded with distinctly less intense running activity.

These results showed that carpenter ants deprived of sensory input from certain of the distal segments of the antennal flagella either did not detect a stimulus, or the stimulus was not sufficiently strong to cause behavioral responses typically given by normal subjects on exposure to x-rays.

The next question concerned the specific kinds of receptors involved. Antennae of ants contain sensory receptors specialized for olfaction, mechanoreception, hygroreception, and thermoreception. While receptors for most senses are also found elsewhere on the body, behavioral studies have shown that, for some hymenopterans, antennal flagella are the exclusive site of olfactory receptors (Schneider, 1964). To test the hypothesis that olfactory receptors were responsible for detection of x-rays by ants, 12 groups of ants were prepared for tests with various odors (n = 3–5). As in the previous experiment, members of each group had "n" segments removed from both flagella, where n = 0 for the intact controls and was increased by one for each succeeding group. Ants were individually presented with the odor of a foreign ant, and then the odor of a nestmate. Results were striking: most ants missing up to nine segments reacted aggressively toward the foreign odor, attacking or attempting to escape from the source. These subjects accepted or ignored the odor of a member from the same nest. In sharp contrast, ants with nine or more flagellar segments missing were not capable of discriminating between the odors of "friend" and "foe." Thus, ants with up to nine segments removed from their antennal flagella responded to x-rays (Fig. 3) and were also capable of odor dis-



Number of Segments Off	Percent Responding
0	100
1	100
2	100
4	90
5	100
6	90
7	90
8	70
9	10
10	20
11	10

FIGURE 3. Latency of the running response as a function of the number of segments amputated from both antennal flagella. Vertical bars show range (n = 10/group). Exposure rate, 10 R/sec. Per cent subjects responding is given in tabular form.

crimination. Those with nine or more segments amputated did not respond to 10 R/sec and could not make the odor discrimination. These results suggest that olfactory receptors participate in the detection of x-rays which result in prompt behavioral reactions of carpenter ants.

Ionizing radiation might have constituted an effective stimulus for flagellar receptors, or acted indirectly via the production of radiolytic byproducts in air which, in turn, stimulated the receptors. An impervious coating of shellac on the antenna should inhibit the potential action of ozone and yet be easily penetrated by x-rays. Accordingly, the antennae of each of 10 ants were shellacked, after which ants were tested for and found incapable of discriminating between odors. Ants were then individually exposed to 10 R/sec for 30 seconds. Seventy per cent of the experimental subjects responded by running behavior within five seconds, but the average latency of response, 1.7 seconds, was twice that of normal subjects. Speed of running was highly variable, and generally weaker than that of controls. These results suggest that behavioral responses were due, at least in part, to x-rays acting in or on the immediate surface of the antennal receptors.

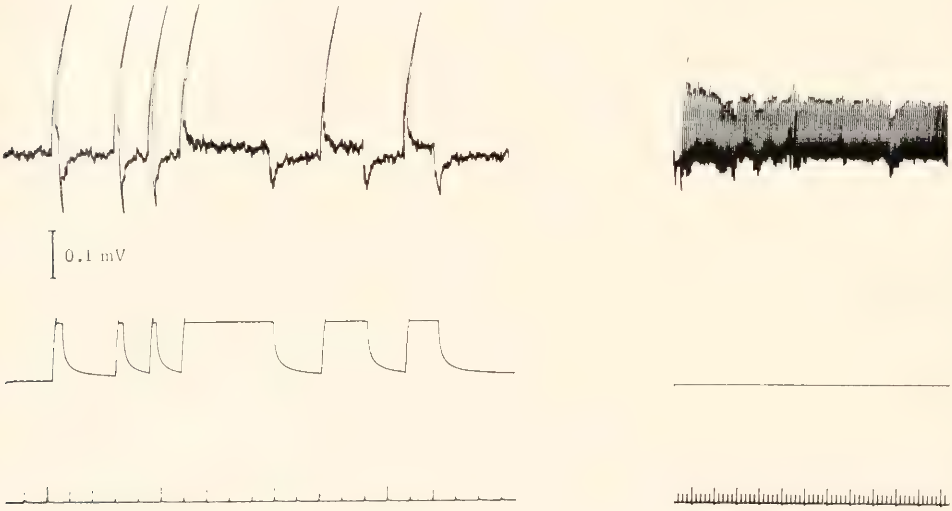


FIGURE 4. ERG response to light (500 nm) stimuli (5.78×10^3 ergs/cm^2/sec) of varying duration (left) and with repeated exposures (40 ms duration) at 2.5 bursts/sec. Top tracing is the ERG response; middle tracing, stimulus monitor; bottom tracing, the time record in seconds. The stimulus marker was inoperative in the right record.

While the responses of light-adapted ants appeared to be mediated entirely by antennal receptors, it seemed possible that dark-adapted ants might respond to x-rays through visual stimulation. To test this hypothesis, 10 antennectomized ants were exposed to 10 R/sec for 20 seconds after the lights in the x-ray room had been dimmed such that the outlines of subjects were barely visible on the TV monitor. No behavioral responses were observed to occur in any subject.

Bioelectric responses of sensory receptors

The isolated eye. The results of behavioral studies illustrated that a visual route of detection need not be postulated to explain prompt behavioral reactions to x-rays. However, the isolated dark-adapted ant eye was found to detect stimuli of ionizing, as well as non-ionizing radiation. Electroretinograms were obtained with light, x-ray, and beta-ray stimuli. Results of the light-induced ERG will be presented first, to provide a base against which responses of the eye to ionizing radiations can be compared.

A rapid increase and decrease in light intensity evoked correspondingly rapid changes in the electrical potential within the eye that are reflected in the ERG (Fig. 4). A slow increase in light intensity did not elicit a response in the ERG, even though the same absolute change in intensity occurred in both cases. With an effective stimulus, regardless of duration, the "on" response (upward deflection) always fell rapidly to the baseline, and cessation of stimulation yielded a deflection of opposite polarity ("off" response). There was a decrease in amplitude of successive responses when the interstimulus interval was less than the complete recovery time of the dark-adapted eye. Also, the strength (amplitude) of the light-induced ERG was a direct function of stimulus intensity.

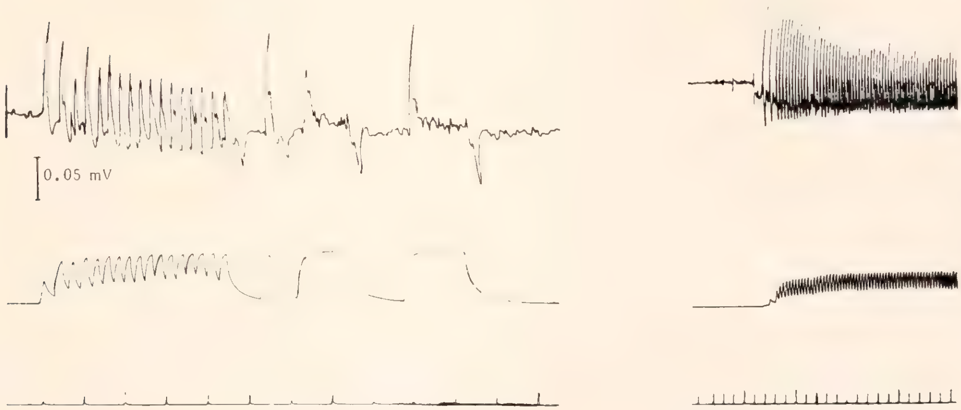


FIGURE 5. ERG responses to a train of x-ray flashes of varying durations (left) and the decrease in response with reduction of interstimulus interval. Tracings as in Figure 4. In right record, flash rate and duration were 3.5/sec and about 1/10 sec respectively. Exposure per flash was 0.22 R.

All eyes exposed to brief x-ray stimuli gave measurable ERGs that were very similar to those elicited by light. Rapid increases and decreases in intensity resulted in sharp "on" and "off" responses, whereas a slowly-changing intensity was ineffective in causing a response. A series of responses to stimuli of various durations are seen in the left part of Figure 5. This record shows that, as with visible light stimuli, x-ray stimulation could modify the responsiveness of the eye to subsequent stimuli. If the interval between exposures was very short, the eye responded at successively reduced strength. Full recovery occurred within one second after termination of a train of rapid stimuli, as manifest by a subsequent response of maximum amplitude (middle of left record, Fig. 5). The right record in Figure 5 shows that the decrease in response with repeated stimulation leveled off within several seconds, after which the eye continued to respond indefinitely, at reduced strength.

The strength of the ERG response was a direct function of the strength of the stimulus, for both x-rays and light. Amplitude-intensity curves for both stimuli exhibit similar slopes through the entire range of intensities used in this study. This and other similarities of x-ray and light-induced ERGs suggests that similar mechanisms might underly the ERG response in the case of both stimuli.

The threshold exposure rate of x-rays to elicit a bioelectric response from the eye was below 300 mR/sec, the lowest exposure rate used in this study. At this rate almost all eyes gave small but distinct peak responses, even when the flash duration was only 100 milliseconds, resulting in an exposure per flash of 30 mR.

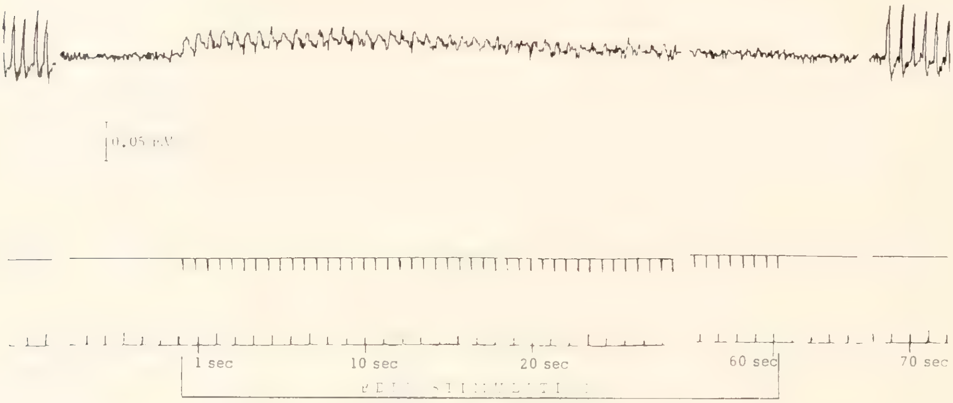


FIGURE 6. ERG responses to beta radiation, preceded and followed by responses to light. As indicated by the interruptions in tracings, sections of the original record were deleted in order to display the series on one line. Beta stimulus conditions were 7.7 mrads per 1/30 sec flash and 1.6 flashes per second. Traces are as in previous records.

Only five of nine eyes tested responded to beta radiation with measurable ERGs. Typical responses to a series of beta stimuli (7.7 mrads/flash) are illustrated in Figure 6. Note that, although the upward deflection of each "on" response rose promptly with the onset of each stimulus, it decayed slowly back to the baseline and there was not a conspicuous "off" response. There was a decrease in the amplitude of responses to succeeding beta stimuli, but this decrease differed from that observed on repeated stimulation with light or x-rays in three ways. (1) The decrease continued until the response peaks merged imperceptably with the noise level of the amplifier and recorder (Fig. 6). (2) Once the eye ceased to give measurable responses to beta stimuli, no further responses to beta stimuli could be elicited, even after periods up to one hour of rest. An obvious assumption was that beta radiation was damaging the eye, but repeated tests showed that such an eye always responded strongly to light stimuli (compare the initial and final light-induced ERGs in Figure 6). Thus beta stimulation, while rendering the eye measurably insensitive to subsequent beta stimuli, did not inactivate the photoreceptive mechanism that was responsive to light. (3) The duration of the interstimulus interval ("rest" period) had no effect on the decrease in response amplitude to successive beta stimuli. If a train of beta stimuli was termi-

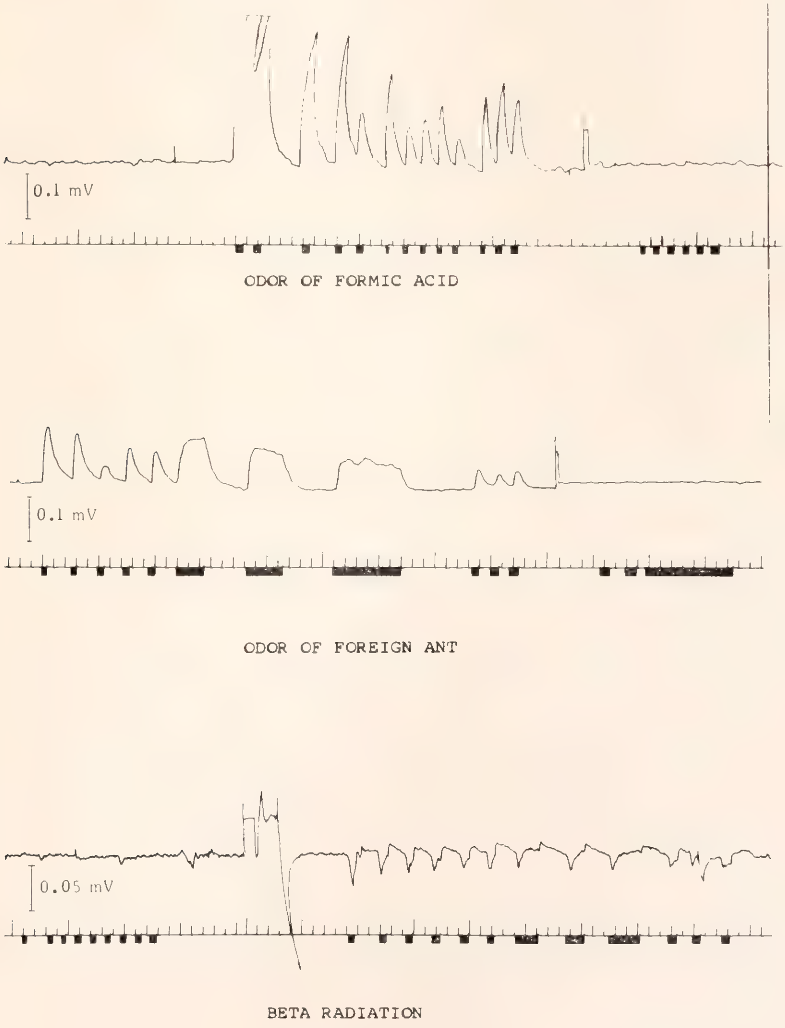


FIGURE 7. Representative EAGs from one isolated antenna exposed to three different stimuli. For each record, upper trace is the EAG, lower trace is the time in seconds. Solid blocks beneath time marks represent the onset and duration of stimuli. Sham tests follow or, in the bottom record precede, the true stimulus. Deflections between exposures and sham tests are artifacts related to operation of the amplifier and recorder.

nated prior to complete loss of responsiveness and then resumed several minutes later, the decline in amplitude progressed as if the two trains had been delivered uninterrupted. Thus, the loss of response to beta stimuli seemed irreversible, and appeared to depend on total absorbed dose. These findings indicate that the mechanisms of action on the eye for beta particles may differ from that for photons in visible and x-ray range.

The threshold dose rate required to elicit an ERG was approximately 62 mrads/sec. At this dose rate, a train of stimuli from the 50 mCi source elicited small but distinct response peaks from one of the eyes studied when the flash duration was 1/20 sec (3.1 mrads/flash).

In spite of the unexplainable differences between x-ray and beta-induced electroretinographic responses, these studies clearly established that, under conditions of dark-adaptation, the eyes of ants detected the presence of high-energy photon and electron stimuli. Such detection, however, apparently did not initiate the prompt behavioral reaction of ants to x-rays.

The isolated antenna. Electroantennograms were obtained from several isolated antennae on exposure to the odor of a foreign ant, the odor of formic acid, and to beta radiation.

Six of eight antennae responded to the odor of formic acid, which is an excitatory pheromone elaborated by ants under natural conditions. The large, upward deflections in the top record in Figure 7 correspond to 400–800 millisecond exposures to this odor. This region of the figure is followed by an illustration that exposure tests to the "odor" of distilled water did not result in responses. An upward deflection means that the tip of the antenna became negative relative to the base. EAGs elicited by the odor of a foreign ant are shown in the middle record of Figure 7. Sham tests on the latter half of the record were again negative. The odor-induced EAGs were monophasic and decayed to the baseline relatively slowly, regardless of stimulus duration.

Four of eight isolated antennae gave an EAG to beta stimuli. The bottom record in Figure 7 shows typical responses to a train of beta flashes of various durations, delivered at a dose rate of approximately 2.2 rads/sec. When compared to odor-induced EAGs, beta responses were of opposite polarity, and response peaks were somewhat sharper, with the decay of each deflection to the baseline beginning immediately, irrespective of stimulus duration. There also appeared to be a slight "off" response of opposite polarity upon cessation of some of the beta stimuli.

The amplitude of response was noted to vary directly with dose rate. Small EAGs to beta stimuli were elicited by 380 mrads/sec, whereas 150 mrads/sec failed to elicit measurable responses, regardless of the duration of exposure. Thus, the threshold dose rate range required to elicit an EAG was between 150 and 380 mrads/sec.

These data offer at least qualitative support for the evidence derived from studies of behavior that sensory receptors on the antennal flagellum can detect a stimulus that could lead to the prompt behavioral reactions of carpenter ants to ionizing radiation.

Discussion

The principal objective of this study was to seek out and characterize behavioral responses given by carpenter ants to bursts of ionizing radiation and to uncover the potential mechanisms through which detection could occur. In this manner, we hoped to extend the body of knowledge on a topic that has been developed almost entirely from intensive studies on selected vertebrate species.

Prompt behavioral responses were found to occur at exposure rates as low as 0.05 R/sec. Characteristics of each response, such as latency, strength, duration, and per cent subjects responding, were strongly exposure-rate dependent. This suggested that excitable tissues were stimulated by something inherent in or accompanying exposure to x-rays.

Any artifactual cue would have had to vary in intensity with the exposure rate. Possible candidates included noise from the x-ray machine and radiation-induced fluorescence in the walls of the exposure chambers. With respect to noise cues, subjects did not respond when the chamber was shielded from x-rays but not from sound. With respect to fluorescence, exposures were made in a well-lighted room in order to mask any potential effects of fluorescence. Another possible stimulus was atmospheric by-products of ionization, such as ozone or oxides of nitrogen. Little work has been done on this problem, since it is difficult to measure the concentration and duration of radiolytic by-products resulting from irradiation of air. However, ants with shellacked antennae did exhibit detection on exposure, suggesting that the effect was mediated within or on the immediate surface of antennal receptors. Thus, none of these concomitants of exposure appeared to be responsible for behavioral reactions to x-ray stimuli.

Previous attempts to identify the physiological mechanisms by which x-ray stimuli elicit behavioral responses from arthropods have implicated photoreceptors. Terwilliger and Levy (1964) discovered that crabs (*Uca*) ceased to give an "off" response when their eyes had been extirpated. Another series of investigations (Smith, Kimeldorf and Hunt 1963; Smith and Kimeldorf, 1964) revealed that various species of moths gave prompt motor responses to ionizing radiation if they were exposed in a dark-adapted state; in a well-illuminated room no responses were observed to occur. These investigators used behavioral and electrophysiological techniques to show that the motor responses of moths were mediated by the dark-adapted visual system, and occurred at exposure rates as low as 10 mR/sec. In contrast to moths, carpenter ants gave motor responses when exposed to x-rays (50 mR/sec) during the light-adapted state. Moreover, bilateral ophthalmectomy did not prevent the initial responses in ants.

Although behavioral responses occurred in the absence of visual detection, the isolated ant eye gave a strong reversible ERG to x-rays. The characteristics of the resulting electroretinogram were very similar to those elicited by light stimuli, suggesting similar mechanisms of action of light and x-rays on the photosensitive pigment molecules in the ommatidia. ERG responses were also evoked by beta radiation at dose rates as low as 62 mrads/sec, with a flash duration of 1/20 sec. Thus, a threshold absorbed dose per flash of only 3.1 mrads was an effective stimulus for the dark-adapted eye. This is somewhat higher than the 0.25 mR exposure required to elicit an ERG response from the eye of the moth, *Pseudaletia unipuncta*, reported by Smith and Kimeldorf (1964). The irreversible decline in responsiveness of the ant eye to a series of beta stimuli has been observed in the eyes of other arthropods (Jordan and Kimeldorf, 1971), but is not understood. Beta-induced ERGs appeared to involve a different or an additional mode of action than that underlying the responses to light and to x-rays.

The occurrence of behavioral responses in light-adapted carpenter ants and in ophthalmectomized subjects indicated the presence of a more relevant detector system. Results of the series of experiments on antennectomized ants leave little doubt that, under the conditions of this study, the principle route by which x-ray stimuli evoked immediate, transient behavior changes in carpenter ants was via receptors (probably olfactory receptors) in some or all of the nine distal segments of the antennal flagella.

Electroantennograms were elicited from isolated antenna by several olfactory stimuli and by ionizing radiation. An understanding of the differences between odor-induced EAGs and those given upon exposure to pulses of radiation must await further study. Detrich Schneider, the worker who has studied extensively the EAG response, reported that the form of the EAG depends on several unknown factors. Different odors, and even the same odor at different times, have resulted in markedly different electroantennograms in silkworm moths, *Bombyx* (Schneider, 1957b, 1962).

The threshold dose rate to elicit an EAG from half of the antennae studied was between 150 and 380 mrad/sec of beta radiations. Within this exposure range (at 200 mR/sec) about half of the ants also gave behavioral evidence of x-ray detection (Figure 1), but the response time was considerably longer for the antennal-waving response than for the appearance of an EAG. Strict quantitative correlations between behavioral and electrophysiological responses are not possible, since different kinds of ionizing radiation were employed as stimuli. Also, the precise relationship between an electrical response and the motor system involved in the behavior is not known as yet.

No previous study has implicated olfactory (or any antennal) receptors in the prompt detection of ionizing radiation by arthropods. As mentioned earlier, olfactory receptors are known to participate in the prompt detection of x-rays by mammals like rats and monkeys (Smith, 1971). Threshold exposure rates for bioelectric responses from the olfactory bulbs of rats (Cooper, 1968), and for behavioral evidence of detection in rats (Garcia, Buchwald, Feder, Koelling and Tedrow, 1964) and in monkeys (Smith and Tucker, 1969) range from three to 50 mR/sec. These rates are lower than the threshold exposure rates found necessary to elicit comparable responses in ants (above), but the important point is that the olfactory system now appears to be initimately involved in prompt responses to ionizing radiation in animals as diverse as ants and primates.

SUMMARY

Carpenter ants exhibited behavioral responses to 10 R/sec (x-rays) within one second of the onset of exposure. Within the range of 0.05 R/sec to 80 R/sec the strength and duration of responses were proportional to the exposure rate. Latency was inversely related to exposure rate.

A comparison of behavioral reactions of normal subjects with subjects whose eyes or antennae had been removed or shielded, revealed that sensory receptors

(probably olfactory) on the antennal flagella were primarily responsible for detection and led to the onset of prompt responses.

Bioelectric potentials were recorded from isolated eyes (electroretinograms) and isolated antennae (electroantennograms) during brief exposures to ionizing radiations. The results largely corroborated evidence derived from behavioral studies that eyes and antennal receptors are sensitive detectors of ionizing radiation. The strength of bioelectric responses varied directly with stimulus strength. Estimates of threshold exposures or doses, delivered in fractions of a second, indicated that the eyes and antennal receptors of the ant can detect x-ray and beta radiations in the millirad dose range.

X-ray and visible-light stimuli elicited electroretinograms with very similar characteristics, suggesting a common mechanism of action on the photoreceptor pigment in the ommatidia. The response to beta radiations displayed some unusual characteristics that cannot be explained at present.

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RESPIRATION AND PHOTOSYNTHESIS IN *CONVOLUTA ROSCOFFENSIS* GRAFF, INFECTED WITH VARIOUS SYMBIONTS[1]

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Measurement of photosynthesis and respiration in algal-invertebrate symbiosis has been used as a means of assessing the functional significance of these associations (for review, see Droop, 1963). Early studies made extensive use of chemical methods for the determination of oxygen produced by the algal partner (Yonge, Yonge, and Nicholls, 1932). The use of polarographic oxygen electrodes offers advantages over the chemical method (Kanwisher, 1959) and this technique has been applied to the symbiosis in hermatypic corals (Kanwisher and Wainwright, 1967; Roffman, 1968), the acoel *Amphiscolops* (Taylor, 1971a) and the sea slug *Tridachia* (Taylor, 1971b). Studies on the symbiosis between the marine flatworm *Convoluta roscoffensis* (Graff) and its green algal symbiont *Platymonas convolutae*, Parke and Manton would be useful for comparative purposes, since the animal appears totally dependent on the algae for its nutritional needs.

The possibility of culturing *C. roscoffensis* in the laboratory (Dorey, 1965), permitted an analysis of the specificity of the symbiosis and led to the finding that a species of another genus, *Prasinocladus marinus* and other *Prasinocladus*-like alge could establish a successful symbiosis with *C. roscoffensis* (Provasoli, Yamasu, and Manton, 1968). The present study attempts to determine the differences in photosynthetic ability of these natural and experimental symbioses.

MATERIALS AND METHODS

Worm and symbionts

Worms used in these experiments belong to three strains collected at Jersey, Roscoff and Hendaye. Continuous cultures were grown in seawater + 2 ml/100 Provasoli's ES enrichment (1968) at 15° C with alternating day/night (14 h/10 h) cycles. The cultures received 500 ft-c illumination provided by cool white fluorescent tubes placed 15–30 cm from culture dishes. Detailed information on the cultivation and maintenance of *C. roscoffensis* in the laboratory will be published elsewhere.

The photosynthetic capabilities of worms infected with different symbionts were examined. These included strains of *P. convolutae* designated as cultures S 6

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[1] Work supported in part by grant AM 10348 of the U. S. Department of Health, Education and Welfare.

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374, W 12 (see Provasoli, Yamasu and Manton, 1968) and Hendaye (from Hendaye worms) and unnatural symbionts, *Prasinocladus marinus* (Plymouth 308), Prasinocladus sp. (S 47), (Parke and Manton, 1965), and *Tetraselmis verrucosa* Butcher (1959).

Measurement of O_2 production and consumption

A Clark polarographic oxygen electrode (Yellow Springs Instrument Co.) connected to a chart recorded (Varian G-1000) was used according to the methods given by Kanwisher (1959). During experiments the electrode was immersed in the medium by inserting it into the side of the glass chamber illustrated in Figure 1. Temperature was maintained at 15° C by circulating constant temperature water (Haake FE circulator) through the outer jacket of the chamber. Illumination was provided by a Sylvania Tungsten Halogen Quartz DWE 150 watt projection lamp connected to a rheostat to give variable light intensities. During experiments light was reflected through the bottom of the chamber by an angled mirror. Side illumination was excluded by the aluminum foil covering part of a cooling water bottle (Fig. 1). Intensity at various rheostat settings was measured in foot-candles with a photometer. Dark periods were achieved by switching off the light and covering the chamber with a double thickness of black cloth.

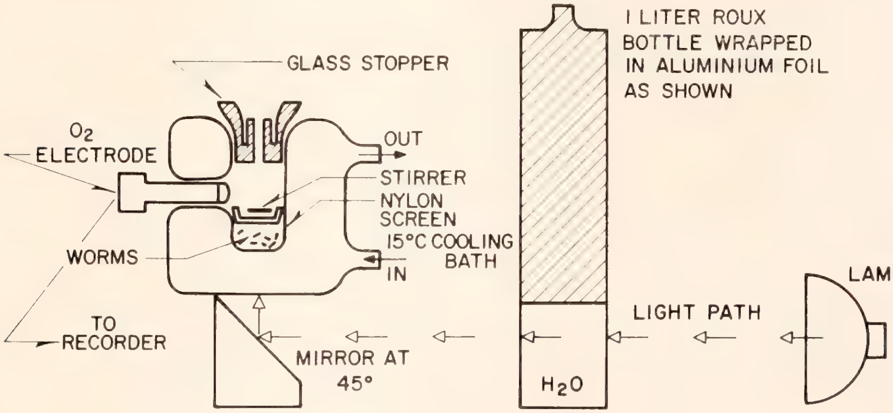

FIGURE 1. Diagrammatic section through the apparatus used for the measurement of oxygen production and consumption. Oxygen determinations are made by a Clark type polarographic electrode (Yellow Springs Instrument Co.). For further details, see text.

For each experiment 10–20 adult worms of uniform size and condition were placed in the well of the chamber which, when stoppered, contained 1.5 ml sterile medium consisting of charcoal treated seawater with 2.0 ml/100 ES enrichment and 0.02 g% $NaHCO_3$. A tight fitting plastic ring with a screen of nylon plankton net was inserted over the worms to confine them to the lower portion of the well and to form a platform for the magnetic stirring bar used to circulate the medium (Fig. 1). One half of the screen's open area (a center stripe) was painted with epoxy glue to reduce the force of water flow in the lower part of the well. Strong flow (even at slowest stirring speeds) disintegrated some worms during these experiments. Following these procedures, the well was covered with a glass stopper and the system allowed to equilibrate for 2–3 minutes before beginning measurements. Experiments usually consisted of consecutive light and dark periods lasting 15 minutes each. Longer time periods (30–60 minutes) were employed for the determination of light compensation points for each symbiont type. At the termination of each experiment, worms were removed from the well, placed in sterile medium and returned to the incubators for 24 hr with a normal light/dark cycle. These were then examined for deaths or abnormalities resulting from the experimental procedure. If none were found, measurements of volume and chlorophyll a content were carried out as described below.

TABLE I

Average photosynthesis/respiration (P/R) values obtained at 1000 foot candles. Each cycle represents one 15 min light period followed by one 10 min dark period. Consecutive cycles were performed on the same group of 10 worms without a break in recording. Calculation of P/R is according to Roffman (1968),

i.e., $\frac{P}{R} = \frac{(P + R)}{R}$.

Host/symbiont	No. of experiments	Cycle	Average net photo $\mu l\ O_2$/hour	Average respiration $\mu l\ O_2$/hour	Average $\frac{P + R}{R}$
Jersey/S 6	6	I	1.48	1.71	1.87
		II	1.70	1.72	1.99
Roscoff/W 12	6	I	2.42	2.64	1.93
		II	2.41	2.68	1.91
Jersey/308	6	I	3.23	3.08	2.05
		II	2.78	3.19	1.87
Jersey/*T. verrucosa*	6	I	1.60	1.88	1.85
		II	1.62	1.84	1.90

Rates of photosynthesis and respiration were calculated by drawing tangents to the recorded tracing at the beginning of the light (photosynthetic rate = P) and dark periods (respiration rate = R) and extending these from 0–100%. The tracing of O_2 production or consumption for 15 min periods was almost always a straight line. Only when the light period lasted longer (~ ½ hour), was a steady decline in O_2 production observed. Gross photosynthesis was taken as the sum of P + R and the ratio gross photosynthesis/respiration was calculated from $(P + R)/R = P/R$ (Roffman, 1968).

Average values derived from these calculations are presented in Table I. In other experiments, the values for net photosynthesis and dark respiration were related to worm volume and chlorophyll a content. Examples of these experiments are presented in Table II. Repeat experiments were qualitatively the same for each individual symbiont type studied. Minor variations in the net rate of photosynthesis between different symbiont types were found to lie within the range of variation encountered with the techniques used.

Worm volume and chlorophyll a content

The total volume of worms in each experiment was determined by centrifugation in capillary tubes. After 24 hr (see above), the worms were pipetted into a micro-hematocrit tube (Clay Adams, Inc., 0.55 mm diameter) open at two ends. One end was sealed with vinyl plastic putty and the capillary was centrifuged for 7 minutes at 12,000 rpm. The length of packed worms was then measured to the nearest 0.1 mm against a scale viewed in a microscope and the volume calculated.

Packed worms were pushed out of the capillary onto a piece of glass filter paper, homogenized with 2 ml of 90/10 acetone/water in a glass tissue homogenizer, and the resulting liquid placed in a conical centrifuge tube. The homogenizer was washed with three 1 ml portions of the acetone/water mix and this added to the extract. After adjusting the volume to 5 ml, the tube was stoppered and covered with Parafilm and aluminum foil. This was stored under refrigeration for 24 hr. The precipitate was then resuspended and centrifuged. The supernatant was decanted and measured in a spectrophotometer at 750, 665, 645, 630 and 480 mμ. Cholorphyll a content was calculated from the formula of Strickland and Parsons (1968).

TABLE II

Oxygen production at varying light intensities

Host	Symbiont	Light (ft-c)	Net photo. $\mu l\ O_2/h$ / mm^3 worm	μg chl.a / mm^3 worm	Net p. / chl.a	Host	Symbiont	Net photo. $\mu l\ O_2/h$ / mm^3 worm	μg chl.a / mm^3 worm	Net p. / chl.a
Hendaye	own	500	1.07	0.76	1.42	Roscoff	*Pr. marinus*	0.72	0.55	1.29
		1000	1.60	0.70	2.30			1.48	0.49	3.10
		2000	1.90	0.81	2.35			1.44	0.30	4.70
		4000	2.54	0.70	3.67			2.47	0.30	8.20
Roscoff	S 6	500	0.53	0.73	0.74	Roscoff	*T. verrucosa*	0.98	0.44	2.21
		1000	1.14	0.62	1.84			1.51	0.50	3.01
		2000	1.88	0.65	2.91			2.23	0.44	5.05
		4000	2.48	0.63	3.94			2.03	0.57	3.56

OBSERVATIONS AND COMMENTS

Table I gives the data obtained from experiments on groups of 10 worms exposed to several consecutive light and dark cycles with the light held constant at 1000 foot candles. Regardless of host strain or symbiont type, the average photosynthesis/respiration value P/R, *i.e.*, $(P + R)/R$, remains nearly the same for all the algae. Within any group of worms, the individual P/R values for consecutive light/dark cycles generally show a progressive decrease with time. This phenomenon is also independent of symbiont type and may be analogous to the progressive decrease in photosynthetic rate observed when groups of worms are kept in the experimental chamber and are exposed to prolonged periods of light (1–1½ hr). Similar effects have been reported previously in symbiotic coelenterates and aquatic ecosystems (Beyers, 1966) and Pacific corals (Roffman, 1968). These authors believed it to be indicative of diurnal variations in photosynthetic rate occurring under normal conditions; but specimen fatigue, photo-destruction of pigments or increased photorespiration could also account for this behavior. Any one of these latter possibilities seems to be a more likely explanation in the present case, since the time periods used here are too short to be indicative of any diurnal changes.

Table II gives typical results of data obtained from experiments relating net photosynthesis and respiration to worm volume and chlorophyll a content when the light is varied from 100 to 4000 ft-c. A different group of worms was used at each light intensity shown to avoid artifacts due to specimen fatigue, *etc.* As in previous experiments, the rate of photosynthesis with all symbionts is approximately the same at similar light values, and is independent of the host strain used. Again, minor variations in net photosynthesis with the different symbiont types, lie within the range of experimental variability. In all cases, oxygen production increases with increasing light intensity up to the level of saturation; this occurs at or near 2000 foot candles. Dark respiration is approximately the same for all experimental symbioses except those involving *T. verrucosa* which appear to exhibit a slightly higher rate.

TABLE III

Average chlorophyll a content (μg/mm^3 *worm*)

Symbiont	Natural symbionts (*P. convolutae*)		Symbiont	Unnatural symbionts (*Prasinocladus*-type pyrenoid)	
	Worm	μg chl.a		Worm	μg chl.a
374	Roscoff	0.83 (21*)	*Prasinocladus marinus* (308)	Roscoff	0.44 (9)
	Jersey	0.65 (6)	*Prasinocladus* sp. (S 47)	Roscoff	0.43 (14)
	Hendaye	0.66 (5)	*Tetraselmis verrucosa*	Roscoff	0.44 (13)
S 6	Roscoff	0.85 (20)			
	Hendaye	0.86 (6)			
	Roscoff**	1.32 (5)			
	Hendaye**	1.10 (8)			
Hendaye	Hendaye	0.69 (19)			

* Number in parenthesis = No. of groups of 10–20 worms analyzed.
** Worms grown at low light intensity (200–300 ft-c).

Important differences in the amount of chlorophyll a/mm^3 worm were observed. Worms reinfected with unnatural symbionts (*Prasinocladus* types or *T. verrucosa*) have approximately $\frac{1}{2}$-$\frac{2}{3}$ the chlorophyll a of worms either bearing their natural symbionts (Hendaye/Hendaye), or reinfected with various strains of *P. convolutae* (S 6). These differences were confirmed and were evident in the average of many experiments (Table III).

As a result, if net photosynthesis is related to chlorophyll a content (Table II, last column) the unnatural symbionts produced more oxygen per unit of pigment than their natural counterparts! To account for these differences the chlorophyll a content of the various algal strains cultured under identical conditions (media

TABLE IV

Characteristics of free living symbionts in vitro

	Average Cell volume (μm^3)	μg chl.a in 10^6 cells
P. convolutae (376)	486	1.7–1.9
P. convolutae (S 6)	486	2.2–2.4
Pr. marinus (308)	1032	2.4–2.6
T. verrucosa	303	2.4
Prasinocladus sp. (S47)		2.5

and light intensity) was measured, but no significant difference was found (Table IV).

Assuming that free-living cells cultured in media have a similar amount of chlorophyll a to cells growing symbiotically, an assumption perhaps unwarranted, then the observed differences would most likely depend on the number of algal cell/worm. An examination of algal cell size, and numbers/worm confirms that this is the case. *P. convolutae* (S 6 and 374) measures 8–13 $\mu \times$ 6–10 $\mu \times$ 4–6 μ (= 192–780 μ^3) (Parke and Manton, 1967) and the average number of cells/worm observed *in situ* was 30,000 resulting in a calculated total algal volume inside the worm of 14.6×10^6 μ^3. The cells of *T. verrucosa* (*Prasinocladus*-type pyrenoid) are somewhat smaller, measuring 8–10 $\mu \times$6–6.5 $\mu \times$ 4.5–6 μ (= 216–390 μ^3) (Butcher, 1959) and are less densely packed to give an observed average of 25,000 cells/worm or a total algal volume inside the worm of 7.6×10^6 μ^3.

TABLE V

O_2 production of experimental symbionts in vitro

Foot candles	Alga	Net photo μl O_2/h	Net photo μg chlorophyll a
	P. convoluta (S 6)		
4000	3×10^5 cells*	12.3	17.1
2000	0.72 μg chlorophyll a	6.4	8.9
1000		5.3	7.4
500		4.2	5.8
	Pr. marinus (308)		
4000	1.8×10^5 cells	9.2	21.0
2000	0.43 μg chlorophyll a	5.2	12.1
1000		3.6	8.4
500		2.5	5.8
	T. verrucosa		
4000	2.5×10^5 cells	11.2	19.4
2000	0.58 μg chlorophyll a	8.2	14.2
1000		6.8	11.7
500		5.4	9.3

* Cell concentrations were selected to give the equivalent # of algae found in 10 worms (see text and Table II).

Clearly, the number of algae/worm has a direct bearing on the pigment content of animals symbiotized with *P. convolutae* and *T. verrucosa*. Among *Prasinocladus* types, the cell size of *P. marinus* (308) is larger than the natural symbiont, measuring 16–20 $\mu \times$ 7–8 $\mu \times$ 7–8 μ (= 784–1280 μ^3) (Parke and Manton, 1965), and averaged 18,000 cells/worm equivalent to an algal volume of 18.6 $\times 10^6$ μ^3. Again, the pigment content of the worms is directly related to the algal number as would be expected.

It is of interest to note that the *P. marinus* symbionts provide the host with nearly $\frac{1}{3}$ increase in the volume of algae, but only $\frac{1}{2}$ the chlorophyll a. The observations on algal number in the living worms were obtained by compressing the worms to a fixed thickness in a Rotocompressor (Biological Institute, 2018 N. Broad St., Philadelphia, Pennsylvania), and were confirmed by the apparent distribution and incidence of algal cells in thick and thin section with the electron microscope.

An examination of algal photosynthesis in culture provides a comparative basis for assessing the performance of experimental symbionts inside the host. The results of measurements on cultured symbionts are given in Table V. Substantial differences in net photosynthesis exist between algae *in vitro* and in the host (*cf.* Tables II and V). This is to be expected since the host's respiration will have a direct effect on the amount of O_2 detected. Other possible contributing factors related to the conditions of a symbiotic habitat could include; (1) increased symbiont photo-respiration, and (2) a reduced or host-regulated photosynthetic rate. Comparisons between experimental symbionts based on the data in Tables II and V show that *P. convolutae* (S 6) and *T. verrucosa* maintain similar levels of O_2 exchange inside the host, *i.e.*, their net photosynthesis in the host (Table II) is approximately 22–24% of the net observed with cultures (Table V). *P. marinus* appears to be capable of maintaining a somewhat higher level of O_2 exchange, *i.e.*, net photosynthesis in the host is approximately 37% of that observed with cultures.

TABLE VI

Compensation points (ft-c)

Natural symbiont (host strain + symb.)		*Unnatural symbiont* (host strain + symb.)	
Roscoff + S 6	100–150	Roscoff + 308	200–250
Roscoff + 374	100–150	Roscoff + *T. verr.*	200–250
Roscoff + W 12	100	Roscoff + S 47	100–150
Hendaye + Hendaye	100	Roscoff + S 1	250
Hendaye + S 6	200–250		
Hendaye + 374	250–300		
Jersey + 374	250		

DISCUSSION

This work was initiated with the hope of exploring the reasons why natural symbionts win out over unnatural ones in the competitive situation of binary reinfections (Provasoli *et al.,* 1968). This success might be explained if natural symbionts could be shown to establish a more effective symbiosis.

No differences in net O_2 exchange were found. P/R ratios and the values for net photosynthesis are consistently similar, regardless of host strain and symbiont type.

This similarity of results is surprising considering the striking differences in the number of symbionts per worm, and the variation in cell size among the symbiotic algae used. There are several possibilities which could explain these results.

Adaptation of the algae to low light intensities used in our cultures of *C. roscoffensis,* may be responsible for the uniform values obtained for net photosynthesis in worms with natural and unnatural symbionts. In nature, *Convoluta* inhabits sloping sand beaches where it is exposed in rivulets of drainage to full natural illumination only during low tide (~ 6 hours/day). Our cultures are maintained at 300–500 ft-c for a photoperiod of 14 hours light/10 hours dark. Despite the wholly artificial conditions, the response may be valuable in showing the great versatility of the symbiosis and its ability to grow under rather unnatural conditions. The apparent uniformity among compensation points for worms infected with a variety of experimental symbionts (Table VI) lends support to the concept of adaptation. However, intrinsic features of the symbiosis itself (see below) could also have an effect, and it seems unwise to rely too heavily on this explanation.

Inspection of electron micrographs reveals clear differences in the way in which different experimental symbionts position themselves in the animal tissues.

Low power electron micrographs show that the more numerous *P. convolutae* protoplasts are closely spaced in the worm tissue, and that they arrange themselves in 3 or more tiers in the dorsal peripheral parenchyma. Those of the *Prasinocladus* species are spaced further apart and do not arrange themselves in definite layers. This suggests that shading will be greater among the more densely packed *Platymonas* than with the loosely arranged *Prasinocladus,* and could account for the lower photosynthetic rates of *P. convolutae* inside the host.

Because respiration, with the chemical and polarographic methods, can only be measured in darkness, an important unknown is introduced in the calculation $P/R = (P + R)/R$ by assuming that dark and light respiration are equal. This assumption disregards the known differences in synthetic and metabolic events typifying the light and dark periods in synchronized cultures of single species of photosynthestic algae (Tamiya, 1966). Applying the P/R relationship to the more complex situation of a symbiosis introduces other unknowns. Are we to assume that the intimate contact between animal and vegetable cells has no influence on either? The method cannot detect variations in the rate of photorespiration of different symbionts due to the host, or variations in host respiration due to the presence and metabolic activity of different symbiont species; photosynthetic rates of symbionts may be host regulated. The uniformity of P/R obtained indicates that interdependencies between animal and vegetable cells are not apparent in the measurement of the total O_2 exchange of the symbiotic unit (host and its algae)—contrasting effects in averaging out may contribute to the uniformity.

A P/R ratio greater than 1 has been regarded lately as indication that the algae are making a positive contribution to the metabolic balance of the symbiosis (Roffman, 1968; Taylor, 1971b) because work with ^{14}C shows that radioactivity accumulates first in the autotrophic symbionts and that the photosynthate migrates readily into animal tissues (Muscatine and Hand, 1958, review of Smith, Muscatine and Lewis, 1969 and Trench, 1971a, 1971b, 1971c).

However, PR ratios very similar to *Convoluta* have been obtained with the symbioses of corals and other marine invertebrates (Kanwisher and Wainwright, 1967; Roffman, 1968; Taylor, 1971a, 1971b). These associations include the complete spectrum of nutritional dependencies, ranging from the facultative (host feeding) symbioses of corals and *Amphiscolops* to the olbigate (host dependent on algae) symbiosis of *C. roscoffensis* examined here. Apparently, the nature of the association and its dependence or independence on an external source of food cannot be detected using P/R ratios or net photosynthesis rates.

At best, a P/R value greater than 1 can be taken to indicate only that excess photosynthate is produced, but it does not indicate its nutritional value to the animal nor that it becomes available to the animal. Muscatine (1971) found that the green algae of 2 sea anemones of the genus *Anthopleura,* photosynthesize (*i.e.*, fix carbon) but do not translocate labeled compounds to the tissue of the host, while the "normal" golden symbionts (*Gymnodinium microadriaticum*) supply their photosynthate to the same species of *Anthopleura*. The real value of the photosynthate produced cannot be assessed without biochemical and nutritional analysis.

While useful data on gas exchange in the symbiosis of *C. roscoffensis* have been obtained, they measure a mass phenomenon, therefore they cannot reveal the competitive advantage of natural symbionts in a situation of binary reinfection, which depends on more subtle and complex events. These may include unequal affinities of the animal cells to different algal species as well as the degree of interdependence and regulation between algal and animal cells.

An additional observation, the degree of intimacy between animal and algal cells, suggests a possible advantage of *P. convolutae* over the *Prasinocladus* species.

During infection, both natural and unnatural symbionts lost their flagella, eye spot and cell wall to become protoplasts (Oschman, 1966; Parke and Manton, 1967). In this condition *P. convolutae* is extremely plastic and is able to position itself between host cells in the peripheral parenchyma, establishing a network of interdigitations with both the animal cells and the muscular fibers. Obviously, the extent of host-algal contact is considerable under these conditions. In contrast, the protoplasts of *Prasinocladus* species do not interdigitate with the host cells, but remain ovate (see Plate I, Fig. 2 and Plate II, Fig. 5, Provasoli *et al.*, 1968). The interdigitation of *P. convolutae* and its greater numbers inside the worm could reasonably militate for a far higher exchange of nutrients (*i.e.*, growth) in worms reinfected with *P. convolutae* than with *Prasinocladus*. This is supported by the results of growth experiments, in which worms reinfected with *Prasinocladus* reach sexual maturity about 10 days later (Provasoli, Yamasu, Mabuchi, in press). However, extensive interdigitation has also been observed in *Platymonas* 315—a very inefficient artificial symbiont (see Plate II, Fig. 4 in Provasoli *et al.*, 1968). Newborn larvae infected with #315 have a very high mortality rate and grow to adult size in 60–100 days instead of 35–50, the usual time for *P. convoluate* and the *Prasinocladus* species-infected worms. High mortality and very slow growth indicate that, despite extensive interdigitation, quantity and/or quality of the products released by the algae are also essential for the welfare of the host.

Summary

Measurements of oxygen balance were made with a recording polarographic electrode on 3 strains of worms reinfected with several natural and unnatural symbionts.

1. The photosynthesis/respiration (P/R) ratios and net photosynthesis varied with light intensity but were independent of worm strains or algal species; compensation light intensities were also similar.

2. However, the content in chlorophyll a/mm^3 of worm varied and in worms reinfected with unnatural symbionts was $\frac{1}{3}$–$\frac{1}{2}$ that found in natural symbiont reinfected worm. Consequently, worms with unnatural symbionts produced more $O_2/\mu g$ chlorophyll a.

3. Cell size of different species of symbiont varied but average chl. a/cell was similar. Observations *in vivo* and with thick and thin EM sections showed that the number of symbionts/mm^3 worm varied with symbiont species. Natural symbionts were more numerous, and more densely packed in superimposed tiers, mutual shading may be responsible for a less efficient photosynthesis.

4. However, adaptation to laboratory light intensities and the assumption implicit in the measurements and calculations of the P/R that dark respiration is equal to photorespiration may also account for the uniformity of results with different symbionts.

5. The P/R ratios appear to be an insensitive way to distinguish obligatory from faculitative symbionts. At best, positive P/R ratios indicate that excess photosynthate is produced but it does not indicate whether or not the photosynthate is released, taken up and/or utilized by the host, nor its nutritional contribution.

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THE ROLE OF CALCIUM ION IN AVIAN MYOGENESIS *IN VITRO*

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The fusion of myoblasts to form a multinucleated myotube exemplifies, albeit in a specialized form, the surface interactions of cells. It is now well-established, by several techniques, that vertebrate skeletal muscle is a *syncytium,* many nuclei lying in a common cytoplasm, and that it is formed by the fusion of mononucleated myoblasts. Until recently all of the evidence was derived from the study of cultured cells (Konigsberg, McElvain, Tootle and Herrmann, 1960; Stockdale and Holtzer, 1961). However, using allophenic mice, crucial evidence has been presented that this process does occur in the intact animal (Mintz and Baker, 1967). Although the fusion of myoblasts is being studied actively in a number of laboratories (Rash and Fambrough, 1972; Konigsberg, 1971; Lipton and Konigsberg, 1972) the process is far from being completely understood. Nor has the mechanism of fusion of animal cells, in general, been resolved, a point of concern in view of the extensive use to which cell fusion techniques are being put in analyses of the biological nature of the cell membrane (Frye and Edidin, 1970) and of cell regulatory mechanisms (Ephrussi and Weiss, 1967).

The hemagglutinating virus of Japan (HVJ, popularly known as the "Sendai virus") readily causes the formation of fused polynuclear giant cells. Okada and Murayama (1966) demonstrated that calcium ions were essential for Sendai virus-induced cell fusion.

Shainberg, Yagil and Yaffe (1970) reported that normal fusion of myoblasts in primary cultures of newborn rat skeletal muscle occurred only when the medium contained at least 1400 μM $CaCl_2$ (which approximates the concentration of Ca^{++} in their complete medium). Observations compatible with these using myoblasts from 12-day-old chick embryos were obtained by Ozawa and Ebert (1971).

In extending these studies of the role of divalent ions in cell fusion, attention has been focused on the effects of Ca^{++} on cell fusion, using skeletal myoblasts from 11-day-old chick embryo thigh. In addition the influence of other alkaline earth cations and Mg^{++} on cell fusion has been examined. A preliminary report has been published (Ozawa, 1971).

MATERIALS AND METHODS

Primary cultures of 11-day-old chick embryo thigh muscle were prepared and incubated overnight. Myoblasts were then purified from contaminating fibroblasts, using the differential adhesion procedure (Kaighn, Ebert and Stott, 1966). This step was repeated twice; thus there were only a small number of fibroblasts remaining at the start of the culture. The collected cells were washed once with Ca^{++}- and Mg^{++}-free Hanks' balanced salt solution, after which they were plated in 30 mm Falcon plastic Petri dishes in which the volume of culture medium was usually 3 ml.

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The medium employed was a modified Ham's F_{12} solution supplemented with 2% embryo extract, 15% horse serum and 5 g of bovine serum albumin per liter. We shall refer to it as medium F_{12215}. In all experiments, following the last differential adhesion treatment, a Ca^{++}- and Mg^{++}-free medium was employed. It was prepared as follows: Chelex-100 cation exchange resins (50–100 mesh) were packed in a column having a diameter of 2.5 cm. The height of the column was about 30 cm per 100 ml of the solutions to be treated, when the resins were in Na^+ form. The resins were washed once with 1 N HCl until the liquid passing through the resins was strongly acidic. At that time, the volume of the resins were reduced. After that the resins were washed with several liters of deionized water, and then washed with 1 N NaOH, until the liquid passing through the column became strongly alkaline. At this time the volume was again increased. The resins were then washed with a large volume of deionized water, until the pH became nearly neutral. Chick embryo extract, horse serum and Ca^{++}- and Mg^{++}-containing reagents were separately passed through the Chelex columns. If denatured protein appeared during the treatment, it was removed by centrifugation. All the components of regular F_{12215} medium, except Ca^{++} and Mg^{++} were then mixed and sterilized by filtration. The medium thus prepared was nominally Ca^{++}- and Mg^{++}-free, and is called Ca^{++}- and Mg^{++}-free F_{12215} medium in this communication. To this medium specified amounts of divalent cations were added to get the medium desired. The osmotic pressure was adjusted with NaCl. When 3×10^{-3} M Mg^{++} was added to this medium, the resulting solution is called Ca^{++}- free F_{12215} medium.

The content of Ca^{++} in the medium was determined by the method described by Yanagisawa (1955).

Most observations were made about 72 hours after cells were plated. If the culture period exceeded three days, media were changed every three days. At the conclusion of the experiment, cells were fixed with 2% neutralized glutaraldehyde and stained by the Feulgen method. Cells were examined by phase contrast microscopy, and the number of nuclei in myoblasts and myotubes were counted. The discrimination of myoblasts and fibroblasts is difficult (Konigsberg, 1963). In this article, long bipolar cells having a sharp border with a strong halo and a weakly stained nucleus are considered to be myoblasts. Only nuclei of myogenic cells, *i.e.*, those of myoblasts and myotubes were counted. Nuclei of other cells, *i.e.*, of fibroblasts or other unclassified cells, were not counted.

RESULTS

The concentration of Ca^{++} in Ca^{++}- and Mg^{++}-"free" medium

Preliminary experiments established that Chelex-100 treatment was essential in the preparation of Ca^{++}- and Mg^{++}-free media; even then traces of Ca^{++} remained. Under the best of conditions, therefore, the media are only nominally "free" of Ca^{++}.

Myoblasts were cultivated overnight in F_{12215} medium, after which they were

TABLE I

Concentration of calcium ion contained in calcium- and magnesium-free F_{12215} medium

Medium number	Treatment	Concentration of calcium ion $\times 10^{-6}$ M
1	Chelex-100	2.2
2	Chelex-100	3.6
3	Dialysis	7.4

washed in Ca^{++}- and Mg^{++}-free Hanks' solution. The cells were collected by centrifugation and the Ca^{++} content of the supernatant fluid determined. It was 8.5×10^{-6} M. When the cells were resuspended and washed a second time, the Ca^{++} content of the second supernatant was 4.5×10^{-6} M. In short, myoblasts themselves "contaminate" the medium with traces of Ca^{++}. Thus it is required that the Ca^{++} content of the medium be as low as possible. In Table I the Ca^{++} content of media prepared by Chelex-100 treatment and dialysis are compared. It is clear that Chelex-100 treatment is the method of choice.

The "baseline" cultures are exemplified by Figure 1. Myoblasts were purified by differential adhesion, washed once with Ca^{++}- and Mg^{++}-free Hanks' solution and plated at 10^5 cells per culture dish in Chelex-100 treated medium. After three days' incubation, most cells had failed to fuse. A few cells formed tiny myotubes, with at most two or three nuclei. Frequently the spindle shape of myoblasts was exaggerated, both poles of the cells being elongated to form long slender arms (Fig. 1).

In short, a myoblast culture containing 10^5 cells might contain about 7×10^{-6} M Ca^{++}, a level sufficient to support abortive myogenesis at best.

Relation between Ca^{++} concentration and myotube formation.

Myoblasts were plated at 10^5 cells per dish in media containing varying concentrations of Ca^{++}, the concentration of Mg^{++} being held constant at 3×10^{-3} M. In judging the effectiveness of a medium in supporting fusion, two parameters were employed: (1) A comparison of the numbers of nuclei contained in myotubes and myoblasts (What fraction of all nuclei counted are in myotubes?); and (2) The size of myotubes, expressed by numbers of nuclei contained in a myotube.

TABLE II

Relation between calcium ion concentration and myotube formation. Cell number plated was 10^5. After three days of incubation, nuclei of myogenic cells in 20 fields were counted at 200 ×

Concentration of Ca ion (M)	Number of nuclei in myotubes	Number of nuclei in myoblasts	Total nuclei in myogenic cells	Percentage of nuclei in myotubes	Number of nuclei in a myotube
3×10^{-3}	535	4	539	99	70–100 in larger ones
10^{-4}	98	26	124	79	usually 5–10
10^{-5}	26	54	80	33	usually 2–3
Nominally free	13	28	41	32	usually 2–3

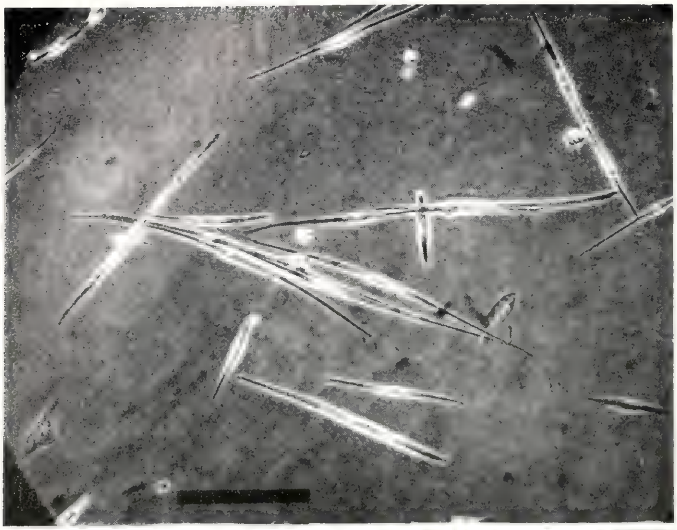


FIGURE 1. Myoblasts plated at 5×10^5 cells and cultivated in Ca^{++}-free medium for three days. Bars in all figures represent 100 microns.

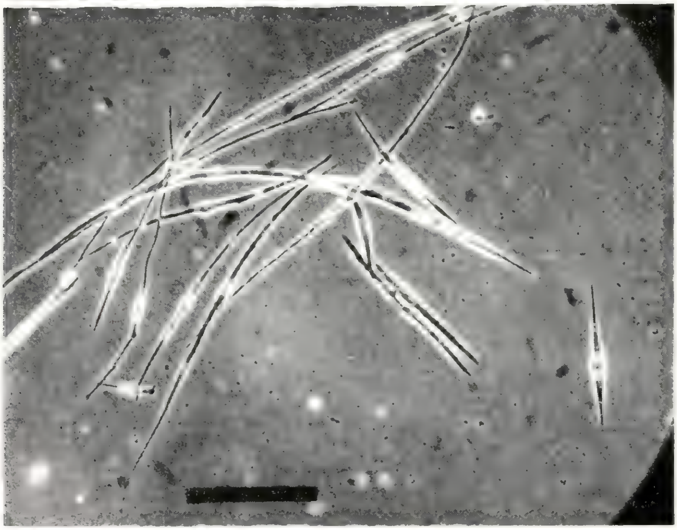
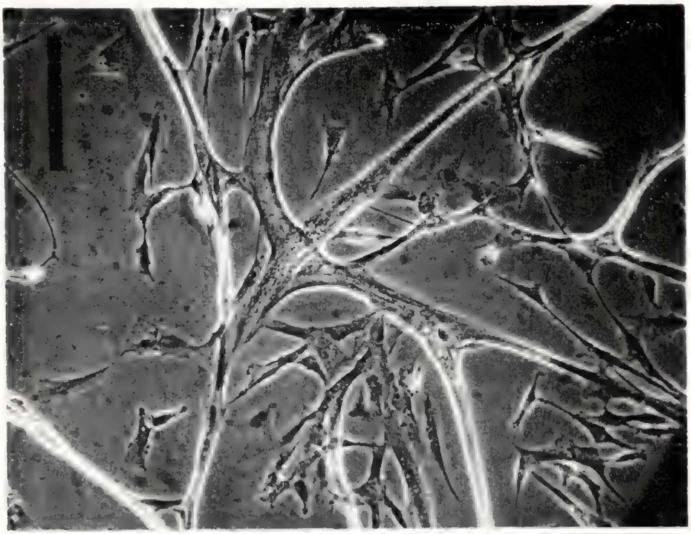


FIGURE 2. Myoblasts plated at 5×10^5 cells and cultivated in a medium containing 10^{-4} M Ca^{++} for three days.

The findings are summarized in Table II. Myogenesis in 10^{-5} M Ca^{++} did not differ appreciably from that in nominally Ca^{++}-free medium. At 10^{-4} M Ca^{++}, the percentage of nuclei in myotubes was increased significantly, but the resultant myotubes were small, containing from 3 to 20 nuclei (Fig. 2). Myotube formation was at its best in 3×10^{-3} M Ca^{++} (Fig. 3). Higher concentrations of Ca^{++} were inhibitory.

Ca^{++} and the fusion process

As can be seen from the column headed "total nuclei in myogenic cells" in Table II, the concentration of calcium ion influenced the number of nuclei in myogenic cells. To exclude the possibility that calcium was influencing a process other than fusion, two additional experiments were performed.



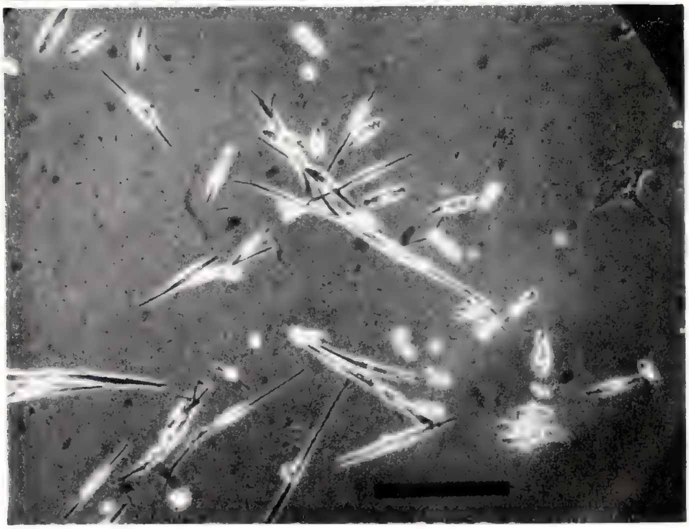


FIGURE 3. Myoblasts plated at 5×10^5 cells and cultivated in a medium containing 3×10^{-3} M Ca^{++} for three days. The figure shows myotubes formed, of which number of nuclei were counted. This figure contains many fibroblasts, of which number of nuclei were not counted.

FIGURE 4. Myoblasts plated at 5×10^5 cells and cultivated in a Ca^{++}-free medium containing 3×10^{-3} M Mg^{++} and 3×10^{-3} M Sr^{++} for three days.

The first concerned the time needed for cell fusion. When 10^5 cells were plated in a medium containing 3×10^{-3} M Ca^{++}, myotubes with a few nuclei appeared after incubation overnight. After two to three days' incubation, the number and size of myotubes increased extensively. When as many as 5×10^5 cells were incubated in C^{++}-free medium for two days, only a few, very small myotubes were formed. However, when this medium was replaced by one containing 3×10^{-3} M Ca^{++}, fusion began within one hour, with myotube formation being extensive after 10 hours.

The second explored the effect of cell population density on cell fusion. As shown in Table III, if 5×10^5 cells were cultured in Ca^{++}-free medium, the total number of nuclei in myogenic cells was 329 after 3 days. However only very small myotubes were formed. This effect can be seen also in Figure 5. On the other hand, when 5×10^4 cells were cultured in Ca^{++}-rich medium, the number of nuclei in myogenic cells was 85 after 3 days. Myotubes with 20 nuclei were observed. As Table III shows, the greater the number of cells plated, the higher the incidence of nuclei in myotubes, and the greater the size of the myotubes formed, providing the medium contains 3×10^{-3} M Ca^{++}.

TABLE III

Relation between cell number and myotube formation. After three days of incubation, nuclei of myogenic cells in 10 fields were counted at 200 ×

Cell Number	Concentration of Ca ion (M)	Number of nuclei in myotubes	Number of nuclei in myoblasts	Total nuclei in myogenic cells	Percentage of nuclei in myotubes	Number of nuclei in a myotube
5×10^5	3×10^{-3}	1090	18	1108	98	70–100 in larger ones
	nominally free	83	243	329	22	usually 2–3
2×10^5	3×10^{-3}	695	16	711	98	40–50 in larger ones
	nominally free	18	51	69	26	usually 2–3
5×10^4	3×10^{-3}	69	16	85	81	20 in larger ones
	nominally free	0	18	18	0	

Effect of other alkaline earth cations on cell fusion

Neither barium nor strontium can replace calcium in its essential role in myoblast fusion.

Cells cultivated in Ca^{++}-free medium containing 3×10^{-3} M Mg^{++} and 3×10^{-3} M Sr^{++} did not behave differently from cells in Ca^{++}-free medium containing Mg^{++} alone (Table IV, Figs. 4, 5). No significant amount of fusion was observed even when cells were maintained in Mg^{++} and Sr^{++} for as long as a week. However when 3×10^{-3} M Ca^{++} was added to such cultures, fusion began at once.

If cells were allowed to multiply in Ca^{++} medium and then transferred to a medium in which Ca^{++} was replaced by Sr^{++}, only tiny myotubes were observed. In short Ca^{++} could not be replaced by Sr^{++}.

Barium was found to be inhibitory. Cells cultured in Ca^{++}-free medium containing 3×10^{-3} M Mg^{++} and 3×10^{-3} M Ba^{++} were lyzed. In lower concentrations of Ba^{++}, *e.g.*, 10^{-4} M, cells appeared to survive, but no fusion was observed. When

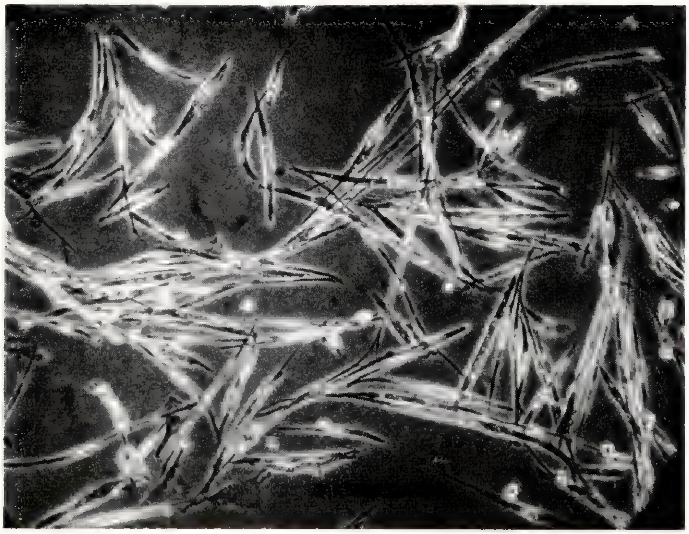


FIGURE 5. Myoblasts crowded in Ca^{++}-free medium do not fuse.

cells were seeded in Ca^{++} medium and then transferred to a medium in which Ca^{++} was replaced by Ba^{++}, they still failed to fuse.

Effects of individual cations on cell fusion

In all the experiments described above, the medium always contained 3×10^{-3} M Mg^{++}. Can Mg^{++} replace Ca^{++}? We now consider cultures in which the medium contained only one divalent cation (Table IV).

Ca^{++} alone is nearly as effective as Ca^{++} and Mg^{++}. When the sole divalent cation is Mg^{++}, even in concentrations as high as 10^{-2} M, significant fusion does not occur.

Are there interactions of Ca^{++}, Mg^{++} and Sr^{++}? This question cannot be answered rigorously. The data suggest that Mg^{++} enhances the effect of Ca^{++} slightly, as judged by the size of myotubes produced, and that by the same criterion Sr^{++} may inhibit Ca^{++} and Mg^{++} slightly.

TABLE IV

Divalent cations and myotube formation. Cell number plated was 10^5; after three days of incubation, nuclei of myogenic cells in 20 fields were counted at 200 ×

Divalent cations added (3×10^{-3} M)	Number of nuclei in myotubes	Number of nuclei in myoblasts	Total nuclei in myogenic cells	Percentage of nuclei in myotubes	Number of nuclei in a myotube
Ca Mg Sr	283	13	296	96	30–50 in larger ones
Ca Mg —	833	16	849	98	100–200 in larger ones
Ca — —	457	12	469	97	70–100 in larger ones
— Mg —	19	57	76	23	5–10
— Mg Sr	10	39	49	20	5–10
— — —	6	24	30	20	2–3

Discussion

The findings show that Ca^{++} plays an essential role in the fusion of myoblasts from chick embryo thigh just as it does in the fusion of myoblasts from the newborn rat (Shainberg, Yagil and Yaffe, 1970). However, according to the latter authors, rat myoblasts *never* fuse in Ca^{++}-free medium; in fact they almost completely fail to fuse in medium containing 2.7×10^{-4} M Ca^{++}, only a small number of myoblasts with two nuclei appearing after 72 hours of incubation. In the nominally Ca^{++}-free medium used in the experiments described herein, about one-eighth of cells tested were found to have two or three nuclei, providing Mg^{++} was supplied sufficiently. If the culture medium contained 10^{-4} M Ca^{++}, myotubes with several nuclei were numerous. Thus chick and rat myoblasts differ in their sensitivity to Ca^{++}.

It was necessary to consider the possibility that the effect on cell fusion was indirect, reflecting another, as yet undisclosed, effect of Ca^{++}. The size of a myotube does depend at least in part on the number of cells available. However, as shown in Figure 5 and Table III, even when cells were crowded, they did not fuse to form large myotubes without Ca^{++}. Furthermore, the brief time needed for fusion after cells were cultured two days without Ca^{++} and then transferred to a Ca^{++}-containing medium, was very short compared with the time required for cell multiplication. Thus Ca^{++} does not influence cell fusion through its effect on cell multiplication.

Ca^{++} cannot be replaced as a requirement for myogenesis by Mg^{++}, Sr^{++} or Ba^{++}, singly or in combination. Nor can neodymium ion, a trivalent cation, which serves in place of Ca^{++} in accelerating the conversion of trypsinogen to trypsin (Darnall and Birnbaum, 1970) replace Ca in promoting fusion (Ozawa, unpublished). Thus myogenesis differs from the aggregation of sponge cells (Humphreys, 1963) and the aggregation of 4-day-old chick embryo limb bud cells (Armstrong, 1966), in which Mg^{++}, but not Sr^{++}, can replace Ca^{++}. Moreover in cell fusion by Sendai virus, Ba^{++}, Sr^{++} and Mn^{++} (but not Mg^{++}) can replace Ca^{++}. In the latter system, the ions are required for membrane reorganization after lysis of virus (Okada and Murayama, 1966).

This study was conducted in consultation with James D. Ebert, who also assisted in preparing the manuscript. The author is grateful for his support. He also wishes to acknowledge the technical assistance of Mrs. Delores Somerville and Mrs. Bessie Smith.

Summary

Calcium ion is required for the fusion of myoblasts from 11-day-old chick embryo thigh, *in vitro*. Calcium cannot be replaced by magnesium, strontium, or barium singly or in combination.

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THE LIFE CYCLE OF *PEACHIA QUINQUECAPITATA,* AN ANEMONE PARASITIC ON MEDUSAE DURING ITS LARVAL DEVELOPMENT

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Peachia quinquecapitata McMurrich (1913) is a burrowing anemone of the family Haloclavidae found on the Pacific coast of North America. McMurrich described the adult *P. quinquecapitata* and also indicated that a larval anemone parasitic on the hydromedusa *Aequorea* probably belonged to this same species. However, since he could not raise one to the adult, non-parasitic stage, he placed it in the genus *Bicidium,* created by Agassiz (1859) for a parasite of a scyphozoan medusa on the Atlantic coast of North America.

Nyholm (1949) discussed the possible dispersal mechanisms available to burrowing anemones such as *Halcampa* and *Peachia.* He cited reports in the literature concerning the development and parasitic habits of *Peachia* and concluded that members of this genus are not obligate parasites, but that eggs or larval anemones attaching to medusae by chance would provide for species dispersal. He noted the paucity of information on the life cycle of this genus, particularly concerning the initiation of parasitism and the nature of the parasitic relationship. The research reported here represents an attempt to provide this information.

Materials and Methods

This research was conducted during the summer of 1967 and the spring and summer of 1970 at the Friday Harbor Laboratories, Friday Harbor, Washington.

The free-living adult specimens of *P. quinquecapitata* were collected by digging in the low intertidal (–1.0 ft or lower) of sandy areas, or by dredging on sandy-mud bottoms. The adults were kept in 12 cm of sand in a sea water table filled to 20 cm with water. They were fed approximately once a week with pieces of shrimp meat or various polychaetes. A fluorescent lamp suspended above the tank provided about 120 foot-candles of light at the water's surface, and was set for 15 hours on and 9 hours off. Water flowing into the tank maintained the temperature at 12–15° C during the summer months.

To induce spawning, the anemones were first kept in the dark for 36–60 hours. After the dark period the tank was uncovered and the anemones returned to the normal light cycle. In addition to the fluorescent lamps, incandescent lamps were also turned on to give about 400 foot-candles at the water's surface. The water flow was stopped for 8 hours each day, increasing the temperature to about 15–18° C. The application of increased light and temperature was continued for a maximum of 15 days or until the anemones spawned.

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The eggs and sperm were collected as they were released. Fertilization and development were allowed to proceed in glass dishes. The sperm were rinsed from the eggs after one hour. The developing embryos were removed to fresh sea water after 12 hours. Subsequently the culture water was changed twice weekly.

Gametes and developing embryos were fixed at various stages for histological and cytological observations. Specimens to be embedded in paraffin were fixed either in Bouin's fluid or Sanfelice's fluid, then dehydrated in a graded series of ethanol and cleared in toluene prior to embedding. Material embedded in Epon was first fixed in 2% glutaraldehyde in Millonig's phosphate buffer (pH 7.4) with the osmolarity adjusted to 970 Milliosmoles. Following the one hour primary fixation the material was post-fixed in 1% Osmium tetroxide in the same buffer for one hour.

The host medusae, *Phialidium gregarium* (Hydrozoa, Campanulariidae), were collected with a dip net from the docks at Friday Harbor Laboratories and kept in jars containing 500 ml of sea water. The jars were partially immersed in a sea table maintaining a temperature of 12–14° C.

The medusae were infected by placing them individually into jars containing 10 to 20 planulae. Alternatively a single planula was pipetted into the stomach of the medusa. Every second day, each medusa and parasite was examined with a dissecting microscope. The parasite was measured and its development noted. To facilitate measurement a small amount of 20% magnesium sulfate was added to the examining dish. This quickly relaxed the medusa. Each medusa was checked for its general condition and the number of gonads remaining. Upon being returned to fresh sea water, the medusa began to swim within two minutes. If the host had little or no gonad tissue remaining, a fresh medusa of the same sex as the original host was put into the culture jar with the host and parasite. The medusae were fed plankton daily.

In 1967 the growth of the parasites was studied from naturally parasitized specimens of *Phialidium gregarium*. These medusae were collected from the docks with a dip net, returned to the laboratory and examined with a dissecting microscope. The medusae having parasites were placed in separate culture jars and checked every second day. In both 1967 and 1970 the rearing experiments were continued until the anemones dropped off the host and became free-living.

Results

Spawning

During the summer of 1967, adult specimens of *Peachia* were kept in the laboratory under normal laboratory light conditions. Attempts to induce spawning by temperature increase and electric shock were unsuccessful and none of these anemones spawned spontaneously. The method of inducing spawning described above was effective, but the time of gamete release varied from 4 to 360 hours after the end of the dark period.

The anemones spawned on 12 separate occasions between March and November 1970. Six of these spawns involved both males and females. Males were observed releasing sperm one-half to two hours before the females released eggs in

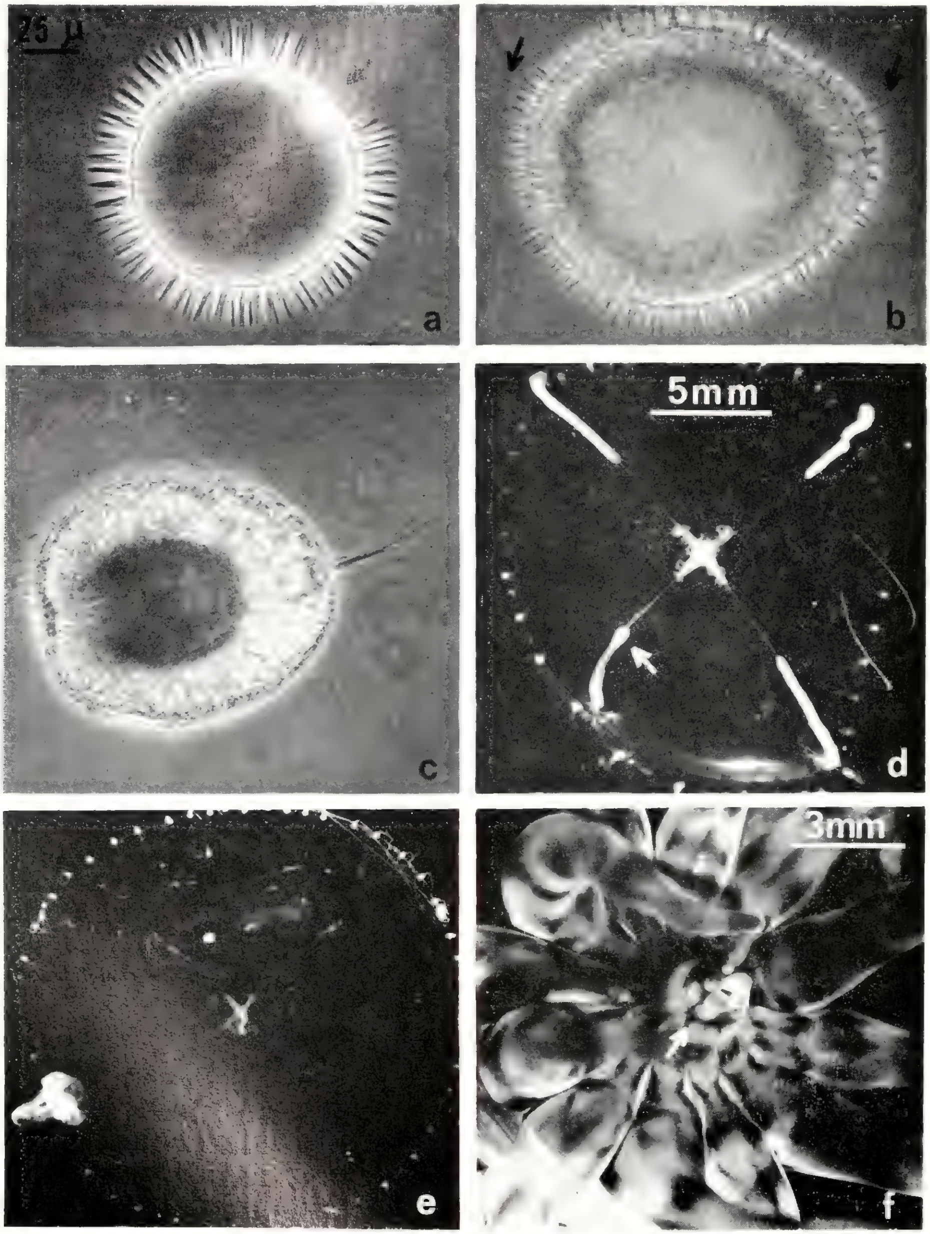

FIGURE 1. Developmental stages of *Peachia quinquecapitata;* (a) The unfertilized egg. The arrow indicates the egg nucleus and the part of the oolemma free of spines; (b) The late blastula stage, Cilia have developed (arrows) and the spines are reduced in length; (c) The planula stage with the characteristic apical tuft. The spines are now gone; (d) Young anemone at the late endoparasitic phase in the proximal part of the gonadal cavity (arrow); (e) later ectoparasite that has 12 tentacle buds; (f) Mature adult showing the details of the mouth (arrow) and conchula.

four of the cases. Two of the spawns were not observed until both sexes were releasing. Of the six spawns involving only one sex, four were by males.

During the time the gametes are being released by *P. quinquecapitata* the anemone pushes up out of its burrow one-third to one-half of its length. In still water the eggs settle close to the female and the mucus released with them rapidly dissipates. The sperm are also released with mucus, which sometimes forms a string before dissipating.

Gametes

The sperm measure 80 to 90 μ in length, the head being about 6.5 by 5.5 μ. The mid-piece is in the form of two swellings which probably contain the mitochondria.

The eggs are 110 μ to 120 μ in diameter and are covered with 20 μ spines, except in the region of the egg nucleus (Fig. 1a). Preliminary electron micrographs indicate that they are extensions of the oolemma. There are no membranes external to the oolemma. The germinal vesicle is broken down to or at the time of spawning. Attempts to fertilize eggs removed from ripe gonads, but still possessing the germinal vesicles were unsuccessful.

TABLE I

Timing of events during the early development of P. quinquecapitata; *water temperature in cultures, 14° C*

Time in hours	Event
0	Fertilization
2	1st cleavage
3	2nd cleavage
4	3rd cleavage
5	4th cleavage
6	5th cleavage
8	Becomes hollow blastula
10	Unfertilized eggs disintegrate
16	Cilia appear between the spines
20	Spines becoming shorter
28	Gastrulation starts
32	Spines no longer visible
40	Apical tuft forms

Development to the planula

At 14° C cleavage begins about two hours after fertilization. Table I gives the timing for various events in development. The early cleavages sometimes appeared irregular at the four-, eight-, and sixteen-cell stages. The blastula is hollow, about 120 μ in diameter, with a lumen of 80 to 85 μ. During the blastula stage the pigment and yolk granules in the cells move to the basal end of the cells. The spines remain until late in the blastula stage. At about 16 hours cilia begin to appear between the bases of the spines (Fig. 1b). Their beating is uncoördinated until 4 to 8 hours have passed. At the time that the cilia begin to appear the spines begin to shorten. Within 8 hours they are reduced in length to about 5 μ, continuing to shorten until they are no longer visible in the light microscope.

Gastrulation occurs by invagination beginning about 28 hours after fertilization. The gastrula is bullet-shaped and swims strongly. The planula stage is reached when the long apical cilia appear (Fig. 1c).

Planulae have been maintained in the laboratory for as long as 30 days in both filtered and non-filtered water. During this time no growth or development was observed. The planulae remained in good condition until about the 28th day when they began to move less vigorously. After 30 days, most were no longer moving and were disintegrating. While in good condition the planulae show a slight positive reaction to light.

Initiation of the parasitic relationship

Field collections of the host medusa, *Phialidium gregarium*, made in 1967 indicated that the smallest parasites were about 120 μ, hollow, had no septa and were located in the stomach or radial canals of the medusae. In addition to the parasites, the stomachs of the medusae contained a number of food items such as eggs and diatoms whose size was similar to that of the smallest parasites. The hypothesis suggested by these data is that the planula or some earlier developmental stage of the anemone is ingested by the medusa during normal feeding, thus initiating the parasitic phase of the anemone's life cycle.

Medusa were put in jars with planulae in 34 initiation trials. Initiation of the parasitic relationship was considered successful if the laral anemone was ingested and survived 24 hours in the gastrovascular cavity of the host. The medusae were examined at various times during the first 24 hour period. At the end of 24 hours planulae were present in 27 of the medusae. In a number of the trials the medusae were watched continuously. Within 10 to 30 minutes after being put into the jar the medusae began to show feeding behavior. Medusae examined after one-half to three hours had one to four planulae in their stomachs, those examined at the end of twelve hours had from one to ten planulae.

To test for possible host-directed swimming by the planulae, isolated stomach-manubrium preparations of *Phialidium* were placed in dishes with planulae. Twelve to twenty-four hours later the stomach contained one to three planulae. In one case a planula was observed as it contacted the oral folds. It remained motionless for a few moments and then was moved into the stomach by cilia of the manubrial epithelium. Isolated stomach-manubrium preparations were put into capillary tubes 5 mm long and with inside diameters of 1.1 or 1.2 mm. Identical empty capillary tubes of the same size were used as controls. In five trials there was no greater density of planulae in or around the capillary containing the medusa tissue.

The first attempts to rear these planulae as parasites in the laboratory after initiation were unsuccessful. The medusae were fed brine shrimp (*Artemia*) prior to and at the time the planulae were in the gastrovascular cavity. Thirty such attempts were made and none of the planulae survived beyond two days. Ten attempts were made with medusae that were starved after the planulae were introduced into the stomach. None of these planulae survived more than two days.

In all subsequent attempts the medusae were fed daily with plankton obtained with a no. 12 plankton net and consisting primarily of arthropods and algae. Under these feeding conditions, the planulae successfully parasitized the medusae. Single planulae were introduced into 65 medusae and of these, 30 lived longer than 2 days. Ten from this group of 30 successful starts completed the parasitic stage and became free-living.

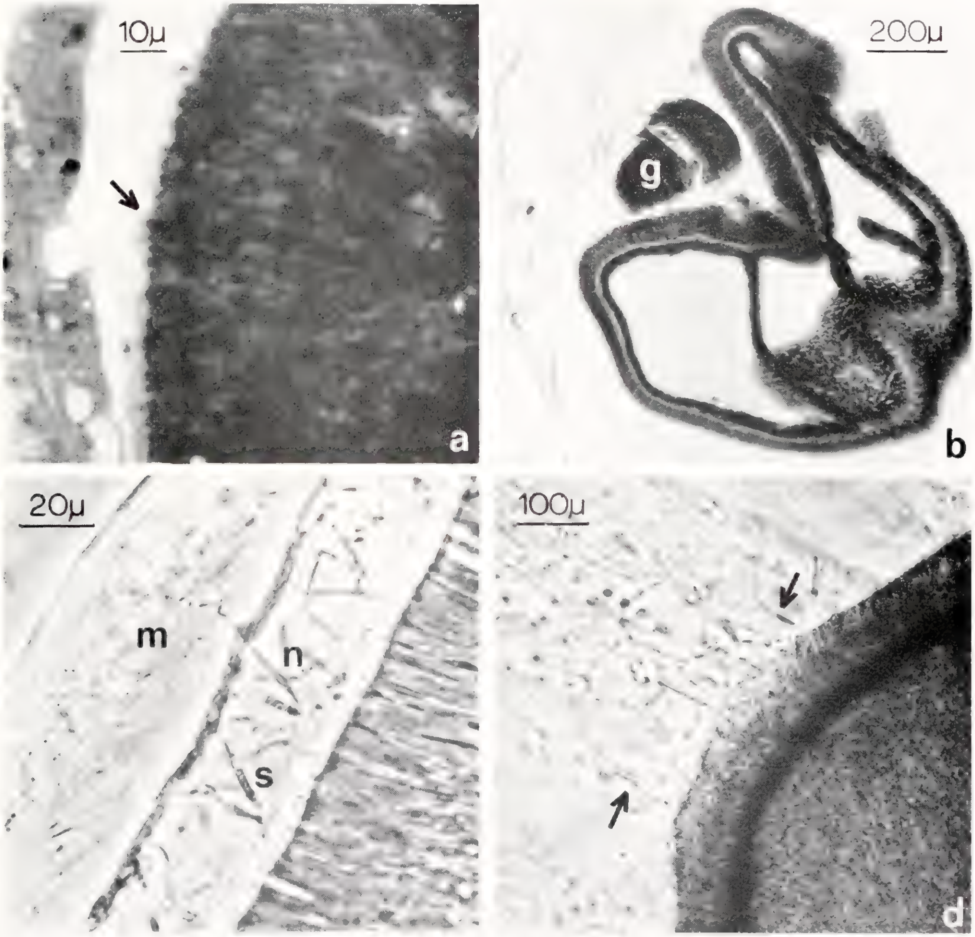

FIGURE 2. Additional details of the parasitism by *Peachia quinquecapitata*. (a) A section of an endoparasite in the radial canal of its host. The arrow indicates the blebbed epidermis of the anemone. Epon section stained with 1% aqueous p-phenylenediamine; (b) Longitudinal section of a parasite on the gonad (g) of the host medusa. Note that the shape of the gonad matches that of the mouth of the parasite. Paraffin section stained with hematoxylin and eosin; (c) Longitudinal section of the oral disk of an ectoparasite attached to the subumbrella of a medusa (m). A nematocyst (n) is shown penetrating the host tissue. Discharged spirocysts (s) are also present. Epon section stained with Azure II, Methylene Blue; (d) A phase contrast micrograph of a tentacle bud showing the discharged nematocysts (arrows) penetrating the subumbrella of the host medusa; a living preparation.

Endoparastic phase

The planulae that survived in plankton-fed medusae began to show a noticeable increase in size by the second to fourth day. The developing larvae remained in the gastrovascular cavity of the host for an average of 11 days in the 1970 rearing experiments (Fig. 1d). They reached an average diameter of 0.5 mm before becoming ectoparasites. Figure 2a is a photomicrograph of a cross section of a parasite in the radial canal of a medusa. The epidermal cells are blebbed, indicating that they are probably obtaining food from the gastrovascular cavity of the host by phagocytosis. The larvae become ectoparasitic either by moving into the stomach and then out the mouth or by "burrowing" through the radial canal tissue.

Ectoparasitic phase

Once outside the host, the parasite moves to a gonad and begins to feed as shown in Figure 1e. A longitudinal section of a parasite on the partially eaten gonad of the host is shown in Figure 2b. Serial sections of this preparation showed complete and untouched gonads in one direction and completely grazed gonad in the other direction. The anemone attached to its host by means of nematocysts during the ectoparasitic phase. Figure 2c shows a cross section of the oral disk of a parasitic anemone with its nematocysts fired into the subumbrella of the host medusa. Figure 2d is a phase contrast micrograph of the tentacle bud of a living *Peachia* attached to its host. The discharged nematocysts are clearly visible.

As the parasites grow their shape changes from that of a flattened sphere to that of a cone with equal length and base diameter. As growth continues the anemone elongates more rapidly than its diameter increases (Fig. 1e). The first eight tentacle buds appear at approximately the 13th day. At this time the anemones averaged 1.5 mm in length and 1.22 mm in oral disk diameter. Four more appear within a short time, making a total of twelve tentacles. The parasites range from dull white to pink in coloration. The first sign of the adult color pattern is the appearance of the light and dark bands on the tentacles. This occurs on approximately the 26th day of the ectoparasitic phase.

The time when the siphonoglyph begins to develop is difficult to determine because the mouth and oral disk of the parasite are held against the host. The smallest anemone on which a siphonoglyph was seen was 0.5 by 0.5 mm. The siphonoglyph in this case was a wide shallow groove. As the parasite increases in size the groove becomes narrower and deeper. Eventually the areas where the siphonoglyph joins the pharynx come together forming the tube-like structure characteristic of the adult. While the anemone is on the host the mouth and part of the pharynx are pressed against the host. This tends to keep the edges of the siphonoglyph from coming together. When the parasite becomes free-living, the oral disc flattens, the pharynx is pulled in, and the edges of the siphonoglyph meet to form a tube.

Bordering the upper margin of the siphonoglyph are papillae which form the conchula (Fig. 1f). They are often not visible until the anemone becomes free-living. They appear first as three small swellings, two lateral and one ventral to the siphonoglyph. Later two more swellings appear between the lateral and ventral papillae.

In the 1970 experiments, the anemones remained ectoparasitc for an average of 31 days. During this time the adult characteristics developed, and they attained an average length of 4.19 mm and a diameter of 2.33 mm. At the end of the ectoparasitic phase the anemones generally released from their hosts, fell to the bottom, and burrowed into the sand if it was present. In a few cases the anemones remained on their hosts, feeding on them until nothing but an epithelial-covered bell remained and it sank to the bottom. The total parasitic stage lasted an average of 41 days in the 1970 experiment.

In 1967 the growth experiment was carried out using anemones obtained as naturally occurring parasites of *Phialadium gregarium*. These host medusae were put into culture before the parasite left the gastrovascular system of the host. In order to compare these data with those of 1970, the day on which the parasites moved from the gastrovascular cavity to the outside was arbitrarily designated as day 11, the average day for this event in 1970. Twenty parasites were reared in the laboratory in 1967. Thirteen of these anemones developed to the free-living stage, four were lost by accidents and three were still parasitic at the end of the time available for the experiment. A comparison of the 1967 and 1970 data was made using the *t* test. This showed no significant difference between their results.

Transfer

Ten attempts were made to carry out the complete parasitic stage on the original host medusa and two were successful. Eight attempts failed owing to death or diseased condition of the host medusae after a long period in culture. In the successful attempts the host medusa were in very poor condition and unable to swim off the bottom at the time the anemones burrowed into the sand. A larval anemone 2 mm in length is able to eat all four gonads off a host in two days. It will then generally proceed to eat the manubrium, stomach and tentacles and, in a few cases, the mesoglea of the bell itself. If given the opportunity under laboratory conditions the parasitic anemone may transfer from one medusa to another.

Numerous transfers have been observed in the laboratory and a few were photographed using a 16 mm motion picture camera. The transfer process involves three stages. (1) Contact between the parasitic anemone and a potential host medusa (Fig. 3a). (2) A period of adherence by nematocysts to both the new potential host and the old original host medusa (Fig. 3b). (3) Release of the original host (Fig. 3c). The complete transfer process from initial contact to release of the old host may take as little as 10 seconds or may last for more than an hour. During a transfer, the anemone is able to hold two actively swimming medusae in spite of very small contact areas (Fig. 3d). In the growth experiments a new medusa was put into the culture dish when it was observed that the old host lacked three or more gonads. After the transfer the old host was removed. In the 1970 experiment the anemones ate an average of 14.6 gonads, involving an average of three transfers. The transfers are possible at all times during the ectoparasitic phase, but are more likely to occur during the early or middle part of the phase. Large parasites (4 to 5 mm in length) show less

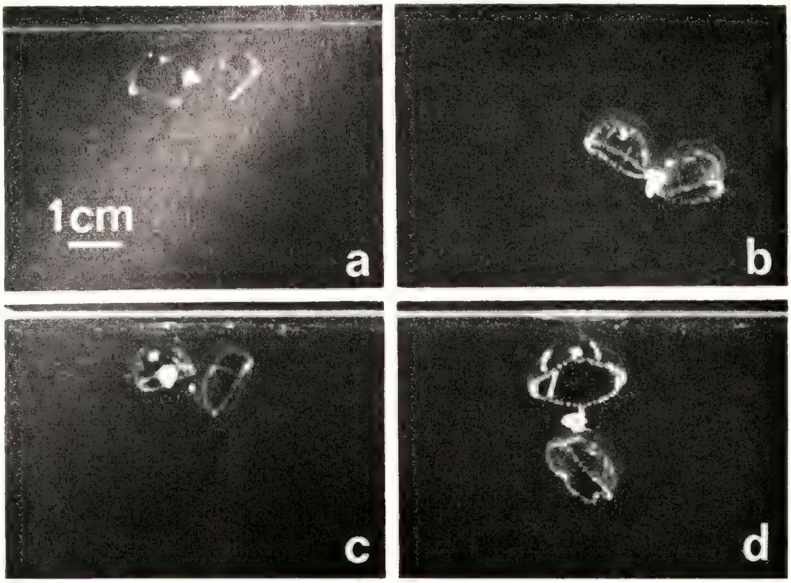

FIGURE 3. Photographs from a 16 mm motion picture record of the transfer by *Peachia quinquecapitata* from one host to another. (a) Two medusae touching so that the parasitic anemone, attached to the medusa on the left, makes contact and adheres to the other medusa; (b) the parasite firmly attached to the two medusae; (c) the parasite just after completing the transfer. The previous host medusa is on the right and the new host is swimming away to the left; (d) a parasite holding a medusa with the tip of one tentacle. The body of the anemone is attached to the original host. Both medusae were swimming during this sequence and the transfer was successful.

tendency to adhere to a new medusa when contact is made and will often remain on their "final" host for a week.

Dip net collections of medusae made in 1967 indicated that the female specimens of *Phialidium* were more heavily parasitized than the males. The hypothesis that the parasite would transfer more readily to a female medusa than a male was tested in a series of 382 tests. In these tests a pair of male and female medusae were placed in the culture jar with a parasite whose host had three or more gonads missing. The pair were chosen to be similar in size and activity on the assumption that the probability of contacting the parasite would be the same. The culture was examined the next day and the sex of the new host noted. Of the 117 successful transfers, 58 were to females and 59 to males. A Chi square value of 0.002 indicates no preference for either sex in transfer. A rank correlation test was run on the 1967 growth data comparing the length of the ectoparasitic phase and proportion of female to male gonads eaten by the parasites. The rank correlation test showed a strong correlation and suggested that those parasites that ate a larger proportion of female gonads in comparison to male gonads became free-living more quickly. Accordingly, in the 1970 study each parasite was restricted to hosts of the same sex as its original host. Thirty such tests were started, fifteen each of males and females. Those restricted to males took an average of 41.2 days for the ectoparasitic phase, ate an average of 15.2 gonads, and were on the average 4.6 mm long when they became free-living. Those restricted to female hosts were ectoparasitic an average of 39.75 days, ate an average of 13.6 gonads, and were on the average 3.7 mm long when they became free-living. Six males and four females survived to the free-living stage. The *t* test applied to these means shows no significant difference between the anemones raised on male or female hosts.

Once off the final host, the anemones burrow quickly into the sand in the manner described by Ansell and Trueman (1968). The average size of all ten animals at the termination of the parasitic stage was 4.19 mm long, 2.25 mm in diameter and with 0.74 mm tentacles. These animals are still being maintained in a sea water table with the adults.

Incidence of parasitism

Records were kept of the percentage of the host medusae, *Phialidium gregarium,* that were parasitized in each dip net collection. The collections made in 1967 showed 8.9% parasitism for the week starting 18 June. The incidence of parasitism increased to 14.1% in the week starting 10 July and declined to a low of 5.0% during the week starting 13 August (the final week of collections). In 1970, collections made on 17 and 19 May contained no parasitized medusae. A collection taken on 2 June had 13.3% parastized medusae. Subsequent collections during June 1970 showed 33.3 to 62.5% parasitized medusae, many of which had endoparasities. Owing to my greater familiarity with the endoparasitic phase, these 1970 records are probably more accurate than those of 1967.

In addition to *Phialidium gregarium* the medusae *P. hemisphaericum, Aequorea aequorea, Halistaura cellularia* and *Mitrocomella polydiodemata* are sometimes parasitized in the Friday Harbor area.

Discussion

The morphology of the anemone used in this life cycle study conforms to the description given by McMurrich (1913) in all but one detail. McMurrich stated that although the central area of the physa in his specimens was very thin, he saw no perforations. Other members of the genus have rows of cinclides on the physa between the insertions of the septa (Carlgren, 1949). During my study, I observed rows of cinclides on the physa of *Peachia quinquecapitata,* in both the free-living adults and the large parasites 5 mm or longer in length. In order that they be visible, the anemone must be very well expanded and propertly illuminated. McMurrich described preserved specimens which were probably not expanded well enough to show the cinclides.

The description of spawning by *Halcampa duodecimcirrata* (Halcampidae) (Nyholm, 1949) is similar to that observed in *P. quinquecapitata.* The major difference is the degree of extension from the burrow. Whereas *H. duodecimcirrata* extends the entire length of its scapus out of the burrow, *P. quinquecapitata* rarely extends more than one-half of the scapus. The data from the laboratory spawnings of *P. quinquecapitata* indicate that most commonly the males begin to release gametes first and that this may stimulate the female to release eggs. This is in general agreement with the observations of Nyholm (1949), but these data should not be taken as conclusive evidence of female stimulation by the male without further experimentation.

The spines of the egg of *Peachia* were first described by Faurot (1895). He felt them to be formed of "vitelline" membrane. The eggs of *Tealia crassicornis* (*Urticina*) are covered by spines (Appellof, 1900), as are the eggs of *Actinia* and *Bolocera* (Gemmill, 1920). Preliminary observations using the electron microscope indicate no extra membranes on the unfertilized egg of *P. quinquecapitata* and no changes have been observed after fertilization viewed with light optics. The function of these spines is not apparent. The eggs of *P. quinquecapitata* are negatively buoyant so their function is not flotation. The spines would function in attachment to the host medusae. However, my observations indicate that by the time the cilia are developed and the larva is swimming, the spines are reduced or have disappeared completely. The pre-swimming stage is short in relation to the planktonic stage (20 hours *vs.* 30 days under laboratory conditions), so that the probability of this being the time of host-parasite interaction is small. In addition I have shown that the initiation of the parasitism takes place readily during the planula phase.

Observations that the medusa of *P. gregarium* feeds upon the planula do not rule out host-seeking behavior on the part of the planulae. In the few experiments made to test for such behavior, none could be found. More elaborate experiments might reveal such behavior, but host-seeking is not a prerequisite to the initiation of parasitism. The tendency of the planulae to seek light would put them into regions where they might be fed upon by the medusae.

During the endoparasitic phase, the anemone is subject to conditions within the gastrovascular cavity of the host. The observation that the larvae would begin to develop in a medusa fed on natural plankton, but not in a starved medusa or one fed on *Artemia* indicates that some specific conditions may be necessary to initiate development. The results of the 1967 experiments, in which the larvae had already begun to develop before brine shrimp were fed to the medusae, argue that once development begins, conditions in the host may be less critical to the parasite.

During the endoparasitic phase the larval anemone appears to derive its nutrition from the partially digested material in the host gastrovascular cavity. The larvae are usually found in the area of the radial canal under the gonad. It is known that partially digested material circulates in the gonadal part of the radial canal (Roosen-Runge, 1967). In addition to food uptake by the gastrodermis of the parasitic anemone, sectioned material shows the epidermis to have characteristics of a phagocytic epithelium. This epithelium changes character during the different phases of the anemone's life cycle, which indicates a change in its function.

Faurot (1895) reported that larvae of *Peachia* obtained from fertilized eggs and cultured in the laboratory died two days after fertilization (probably at the planula stage). I was able to keep planulae alive for up to 30 days after fertilization, but with no evidence of growth or development. Planulae which were put into a host medusa began to grow and differentiate within one or two days. This indicates that a period of time in the gastrovascular system of a medusa may be necessary for the initiation of development. Such situations are not uncommon in gut parasites such as nematodes which have been shown to need specific stimulae to trigger hatching or development (Rogers, 1960). The fact that *Peachia* planulae did not grow or differentiate in my cultures is not proof of an obligate parasitism, but it argues strongly in favor of this hypothesis.

During the ectoparasitic phase, the larvae of *P. quinquecapitata* feed on the gonads and other body tissues of the host medusa. Earlier authors have reported different observations on other species of *Peachia*. In most cases the term parasite has been used in a very general sense. Müller (1860, page 435) reported that the parasitic anemones he had collected contained "fragments of tentacles, filaments, genitalia, stomachal filaments, etc." in their gastrovascular cavities. However, he noted that the parasites could go for months without food and would eat things other than medusae. Haddon (1887) fed the parasites small pieces of meat when no medusae were available. He stated that the medusa was probably killed by the parasite, but gave no evidence for this. I have maintained a few larvae of *P. quinquecepitata* on shrimp and clam meat, but none ever completed development to the adult stage. McIntosh (1887) stated that it was not necessary to limit the parasite to feeding on medusae. He suggested that the anemone could capture food with its mouth while remaining attached to the medusa with tentacles. This type of behavior was not observed in my study of *P. quinquecapitata*. Browne (1896) described *Peachia* on the gonads of the hydromedusa *Phialidium*, but did not report whether they were feeding on the gonads.

Badham (1917) describes the tubular siphonoglyph and conchula as larval structures functional in feeding. He describes a constant stream of water passing into the conchula and out through the physal cinclides of parasites 5 to 40 mm in length. It should be pointed out that the species which he studied (*P. hilli*) was living inside the radial canals of a large rhizostome medusa, *Crambessa mossica*. Panikkar (1938) describes a stream of water passing into the conchula of *Metapeachia tropica* (*P. tropica*) attached to the subumbrellar surface of *Aequorea pensile*. He noted that until the conchula and siphonoglyph were developed, no food was seen in the gastrovascular cavity. After these structures developed, diatoms and copepods were always found in the gastrovascular cavity of the parasite. I have observed pores in the physa of parasites over 5 mm in length, and although a stream of water was observed to pass into the siphonoglyph in a few cases, no particles were seen to pass into the anemone. This was not observed in smaller anemones and many became free-living before the pores become noticeably differentiated. It is possible that *P. quinquecapitata* supplements its diet of gonad with food carried in by ciliary currents, but I have not observed it.

Transfer behavior has not been described for *Peachia* before, Ross (1967) has described the transfer behavior of the anemones *Calliactis, Stomphia, Actinostola* and others toward mollusc shells with which they are associated. These transfers involve initial sensing of the shell by the anemone through tentacle contacts. This is followed by adhesion of the tentacles through nematocyst discharge and a detachment of the base of the anemone. Movements then bring it into contact with the shell to which the tentacles are attached and the tentacles are released. The transfer of *P. quinquecapitata* does not involve the active movement of the anemone from one host to another but does involve recognition and attachment by the discharge of nematocysts and adherence to the new host. This is followed by a differential release of nematocysts which results in the release of the old host and continued adhesion to the new host. The actual separation from the old host is accomplished through the swimming of the medusae and not movement of the larva.

This transfer has not been observed in nature, but it reasoned to occur there. Most medusae collected with a parasite are missing one or more gonads. Occasionally a medusa is collected bearing a parasite 2 mm or longer, but still having all four gonads intact. It is presumed that in these cases transfer had recently occurred. In cases where such infected medusae were kept in the laboratory the parasites began to eat the gonads within a very short time.

It has been demonstrated that a parasite can develop to the free-living stage on the original host and that transfer is not necessary. The hosts in these cases were essentially dead at the end of the parasitism. Transfer to a new host would provide better nutrition and transportation for the parasite and preserve the life of the host. It has been shown by Roosen-Runge (1965) that the medusa *Phialidium* can regenerate gonads within 14 days after their surgical removal.

Phialidium gregarium occurs in dense swarms in the Friday Harbor area and although no quantitative measurements have been made to determine densities, I have observed contacts between medusa. I hypothesize that contacts occur frequently enough to permit transfers of the larvae.

Contrary to Nyholm (1949), I conclude that *P. quinquecapitata* is an obligate parasite for at least a short period during its development. The apparent inability of the free-living planula to continue development, the specialized feeding behavior as an ectoparasite, and the transfer behavior are adaptations which seem well suited to a period of parasitic life. Other species in this genus may not parasitize their hosts in the same way as *P. quinquecapitata* or may not be obligate parasites. Answers to these questions await more detailed studies of the other species.

I wish to thank Dr. R. L. Fernald, Director of the Friday Harbor Laboratories, University of Washington, and Dr. L. A. Fraser, University of Wisconsin for support of this work, and my wife Karen for help in collecting and rearing the anemones. This research was supported by a grant from NSF to the Friday Harbor Laboratories during the summer of 1967 and by NIH Developmental Biology Training Grant HD 00266-03 to the University of Washington.

SUMMARY

1. *Peachia quinquecapitata* kept in sea water tanks at the Friday Harbor Laboratories were induced to spawn. The egg is 120 μ in diameter and is covered with spines 20 μ in length.

2. Cleavage leads to a hollow blastula which becomes ciliated. As the cilia appear the spines become shorter and disappear.

3. Gastrulation is by invagination. The planula stage is reached when the gastrula develops an apical tuft of long cilia.

4. A swimming planula can be ingested by the medusa *Phialidium gregarium*. After ingestion the larva begins to grow and differentiate. Planulae which did not get ingested would not develop in the laboratory.

5. The larvae remained endoparasitic for an average of 11 days and probably were feeding on material in the gatrovascular cavity of the host medusa.

6. After 11 days the larvae became ectoparasitic and fed on the gonads of the host medusa. After an average of 31 days of ectoparasitism anemones had acquired their adult characteristics and dropped off the host medusa to become free-living.

7. During the ectoparasitic phase the larval anemones could transfer from one host medusa to another in the laboratory cultures. Transfer has not been observed in nature but is reasoned to occur there.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

Abstracts are arranged alphabetically by first author. Author and subject references will also be found in the regular volume index, appearing in the December issue.

GENERAL SCIENTIFIC MEETINGS

August 21–23, 1972

Further evidence for potassium concentration changes in the periaxonal space of the squid giant axon. W. J. Adelman, Jr. and J. P. Senft.

The purpose of this work was to show that there are apparent changes in [K] immediately adjacent to the excitable membrane that are not related to external sodium ions. This was done by producing outward potassium current flow into an external sodium-free solution containing high K. Squid axons were externally perfused with an artificial sea water (ASW) with zero [Na], 340 mM [K], 10 mM [Ca], and 50 mM [Mg], and made isosmotic to sea water by addition of TrisCl. In this solution, the resting potential, E_{RP}, ranged between +1 and −1 mV. Axons were voltage clamped with the holding potential $= E_{RP}$. In clamp, axons were pulsed with sets of two successive pulses of amplitudes E_1 and E_2. For E_1 values of +50, +60, and +100 mV, I_m jumped to large outward values as expected, rapidly increased slightly, and then slowly declined or "drooped." The duration of these potassium currents was varied. For each duration (from 1 to 40 msec), the instantaneous current-voltage relation was determined from initial values of the tail currents for a set of E_2 values. These I/V relations were quite linear. From their slopes and zero current axis crossover voltages, $g_K(t)$ and $E_K(t)$, respectively, were obtained. As I_K duration increased, E_K became more positive with a time constant of about 10 msec. As the leakage conductance was negligible (about 0.4 mmho/cm^2), these E_K changes were taken as indicative of rapid current mediated changes in either internal or external [K]. The kinetics of the change in the internal to external [K] ratio was calculated from $E_K(t)$ values as a function of E_m. If these changes occur because of potassium ion accumulation in the periaxonal space resulting from limited K^+ diffusion across the Schwann cell layer barrier, then, in the high external [K] case, [K] increases in the space from 340 mM to about 540 mM during a 30 msec outward I_K of 1 mAmp/cm^2. These experiments support the theory that apparent E_K changes during outward I_K flow through axon membranes result from [K] changes in regions close to the membrane rather than from shifts in reversal potentials resulting from contributions of other cationic charge carriers than K^+ to the potassium current.

Limulus polyphemus: *a true Cyclops.* Robert B. Barlow, Jr. and Ehud Kaplan.

A horseshoe crab (30 cm female) with an extra pair of compound eyes was found by Paul Shave in the summer of 1972 near Orleans on Cape Cod. The extra eyes are situated at the top of a chitinous stalk (2 cm high and 0.5 cm in diameter) which is located on the dorsal surface of the carapace three centimeters off the midline. The lateral and median eyes and other parts of the animal appear to be normally formed.

Electroretinograms recorded from the "stalk eyes" are similar to those recorded from the lateral eyes of this animal. Optic nerve activity from the stalk eyes was detected through wick electrodes placed on the external surface of the shell. This optic nerve activity can influence the animal's behavior as demonstrated by measurements of the heart rate. In normal specimens of *Limulus* illumination of either of the lateral eyes increases the heart rate and cessation of the illumination decreases the heart rate. (R. B. Barlow, Jr. and T. Palfai, unpublished observations). Illumination of the stalk eyes produced the same effects. The neuroanatomy of the visual system of this animal has not been studied since the animal has not as yet been sacrificed.

To our knowledge this is the only horseshoe crab found with an extra pair of compound eyes.

Pigment granule movements in Arbacia *eggs treated with cytochalasin B and colcemid.* Ann M. Belanger and Ronald C. Rustad.

The echinochrome pigment granules of the eggs of *Arbacia punctulata* are known to exhibit random saltatory movements in unfertilized eggs, migrate to the cortex at fertilization and begin leaving the region which will ultimately form the surface of the four micromeres as early as the two-cell stage.

We have previously shown that these movements are inhibited by cytochalasin B, but are unaffected by colcemid. Prolonged exposure of fertilized eggs to cytochalasin B often resulted in surface wrinkling, hypertrophy of the hyaline layer, aggregation or lysis of pigment granules and extrusion of these granules into the hyaline layer. Hence, cytochalasin may affect either the plasma membrane or the cortical layer of the egg.

The movement of pigment granules out of the presumptive micromere region of eggs treated with colcemid was filmed over a 40 minute period (2 frames/minute), during which time approximately 50% of the original granules left the region. The granules exhibited no long saltations. Occasional displacements of up to 3 μ/minute were observed; however, granules generally moved 1 μ/minute or less. Granules originally at the edge of the region tended to disappear from view first, followed by those more centrally located.

While these observations have not established whether this movement is saltatory in nature, they have clearly demonstrated a directed movement of pigment granules away from the presumptive micromere region.

This research was supported by Contract W-31-109-ENG-78 with the U. S. Atomic Energy Commission, and by an N. I. H. Training Grant.

The distribution of genetic variation in three species of Littorina. Edward M. Berger.

Gene-enzyme variation was examined electrophoretically at three non-specific esterase loci, in 14 sympatric populations of the prosobranch gastropods: *Littorina littorea, L. saxatilis,* and *L. obtusata.* Allele frequency data for these polymorphic loci revealed striking interpopulation heterogeneity in the species *L. saxatilis* and *L. obtusata,* but a marked homogeneity between populations of *L. littorea.* A method for calculating an index of interpopulation heterogeneity, $H_{a.b.}$, was developed and applied to the data. The values calculated for *L. saxatilis* and *L. obtusata* were similar, but 3 to 4 times greater than estimates for *L. littorea.* These differences are discussed with respect to the divergent dispersal capabilities which have evolved in these species.

This work was supported by a grant (GM 18910) from the NIH.

Disc-gel electrophoresis of fish hemoglobins. Thomas A. Borgese.

Polyacrylamide disc-gel electrophoresis of oxyhemoglobins from the skate (*Raja erinacea*), sea robin (*Prionotus carolinus*) and killifish (*Fundulus heteroclitus*) were compared using a Tris-glycine buffer pH 9.0. Multiple hemoglobins were observed in all species following electrophoresis at 4° C and 150 volts for two to four hours. Six hemoglobin bands (labeled I through VI in the order of their increasing anodal mobility) were found in the skate and four and three bands were observed in the killifish and sea robin, respectively. Hemoglobin proportions, determined with a Photovolt densitometer and integrator, gave mean values of 5, 13, 28, 34, 15 and 5% for skate hemoglobins in the order of increasing anodal mobility. The range of values for each was 3–8, 12–13, 24–32, 30–37, 12–18 and 4–7%, respectively. For *Fundulus* the proportions were 13, 36, 36 and 15% with the following range; Hb I (9–17), Hb II (31–39), Hb III (31–38) and Hb IV (12–18). In replicate experiments, sea robin Hb I gave a mean value of 8% (7–8), Hb II 36% (33–39) and Hb III 55% (54–59). Iso-electric focusing with pH gradients of 5–10 (50 volts), and 7–10 (125 volts) for 4 to 6 hours produced similar hemoglobin patterns as the more conventional electrophoresis. Additional minor bands, and increased diffuseness of previously sharp bands, were observed on continuing iso-electric focusing for a total of 22 hours in several different species including toadfish, tautog and dogfish. Additional hemoglobin bands were also observed during storage at 4° C. Both *Fundulus* and sea robin have in excess of 5 hemoglobin bands after storage from one day to six weeks. It is likely that the effects observed with prolonged electrophoresis and storage represent *in vitro* modifications of the structure of the original hemoglobins.

Changes in $[Ca^{++}]_{in}$ *of* Limulus *ventral photoreceptors measured with aequorin.* J. E. Brown and J. R. Blinks.

Aequorin dissolved in 0.5 M KCl was pressure-injected intracellularly through a micropipette. During the receptor potential elicited by either brief or prolonged stimuli, a transient aequorin luminescence occurred. The aequorin luminescence occurred with the membrane voltage-clamped; at reversal voltage for the light-induced current, the aequorin luminescence was about $\frac{1}{2}$ as large as at dark, resting voltage. The aequorin luminescence evoked by a fixed size stimulus, was larger in 4× Ca^{++}-SW and was smaller in O Ca^{++}-SW than in ASW, was graded in size with stimulus intensity, and had no measurable plateau component using prolonged stimuli as bright as 35 $\mu W/cm^2$ on the photoreceptor. The transient aequorin luminescence fell with half-times as short as 200 msec; with very bright, prolonged stimuli, $T_{\frac{1}{2}} \approx 600$ msec. To the contrary, in photoreceptors of *Balanus eburneus,* the aequorin luminescence had both transient and plateau phases graded with stimulus intensity; the $T_{\frac{1}{2}}$ for the decay of the transient could exceed 600 msec. We propose that in *Limulus* ventral photoreceptors, during the light response the $[Ca^{++}]_{in}$ rises (with a delay) and is actively removed to a level less than 10 times its peak concentration. The rise in $[Ca^{++}]_{in}$ is probably due only in part to a light-induced increase in calcium conductance.

This work was supported by NIH grants: EY-00834, EY-00835, EY-00897 and by HSAA RR 06067.

Delay of fertilization in Arbacia *by cytochalasin B treatment.* Robert F. Brunhouse, Phoebe Mounts and Dennis Barrett.

Cytochalasin B, a reagent postulated to exert specific effects on processes mediated by cytoplasmic microfilaments, was used to treat suspensions of 2×10^8 *Arbacia punctulata* sperm per milliliter. When insemination was carried out in the continued presence of high cytochalasin B (*e.g.*, 5 μg/ml) fertilization was blocked. If, instead, the inhibitor was diluted 100-fold at insemination (*e.g.*, to 50 ng/ml) fertilization ensued (as determined by the appearance of the fertilization envelope), but was consistently delayed. The delay is thought to reflect the time required for the cytochalasin B concentration to be reduced by diffusion, to a level where the sperm can function.

The delay resulted from treatment of the sperm before insemination, since the final dilution of cytochalasin B caused no delay when added to eggs and sperm at the time of insemination. Cytochalasin B was diluted from stock solutions in dimethylsulfoxide, but controls showed no effect of the solvent in the highest concentration used, 0.1 per cent.

The magnitude of the delay was proportional to the concentration of cytochalasin B during sperm pretreatment. Statistically significant delays resulted from as little as 2 μg/ml. The reagent exerts its effect on the sperm very rapidly: 30 seconds' treatment (at 5 μg/ml) causes maximal delay.

Cytochalasin B delays fertilization without affecting the motility of sperm. We hypothesize that it acts through some component of the acrosome reaction.

This investigation was supported by PHS Training Grant No. GM00265–14 from National Institute of General Medical Science.

Two different pools of hexosaminoglycans in the corneal stroma of the dogfish. G. CREMER-BARTELS AND Z. DISCHE.

It has been shown by G. Key and Z. Dische that the bovine corneal stroma contains two fractions of hexosaminoglycans, one extractable by 0.15 M NaCl at pH 6.0–6.4 and another one which is extracted only by 1 M $CaCl_2$ at pH 8.0. Stromal hexosaminoglycans are believed to be related to the swelling of the excised bovine cornea in water and salt solutions. Corneas of elasmobranchs do not swell under these conditions. It seemed possible that only one of the two fractions of hexosaminoglycans of the bovine cornea is involved in its swelling. If this were the case it could be expected that no difference will appear in the extraction of dogfish corneas by NaCl pH 6.3 and $CaCl_2$ pH 8.0, respectively.

Batches of 12 corneas from dogfishes of different ages (weight varying between 33 and 67 mg) were removed immediately after the death of the animal, stripped of the epithelium, and incubated with stirring at 5° C for 16 hr in elasmobranch Ringer's solution in presence of antibiotics. The extraction mixture was then centrifuged and the supernatant dialyzed first for 24 hr against running tap water at 2° C and then against large volumes, once changed, of distilled water for 48 hr. The extraction residue was twice more subjected to the same procedure. The dialysates were centrifuged to separate the extracted collagen which precipitated during dialysis and the supernatants were analyzed for hexosamine, hydroxyproline and hexose. Residue from the Ringer's extraction was re-extracted in an identical way with 1 M $CaCl_2$ pH 8.0 and the extracts treated and analyzed. The amount of hexosamine extracted by the Ringer's decreased sharply with each following NaCl extraction and went sharply up in the following $CaCl_3$ extract. The hexosaminoglycans of the dogfish cornea do not show a significant difference in their extractability by salts from those of the bovine cornea.

Supported by: Grant CA 02075 of The National Cancer Institute and a research stipend Cr/40 of the Deutsche Forschunges Gemeinschaft.

Changes in fluorescence of squid axons during activity. H. V. DAVILA, B. M. SALZBERG, L. B. COHEN AND A. S. WAGGONER.

The physical properties of many fluorescent molecules are influenced by, and therefore reflect their immediate surroundings, transmitting information about their microenvironment through characteristic features of their fluorescence spectra.

More than 150 fluorescent dyes were applied to the giant axon of the squid, *Loligo pealei,* and their fluorescence was monitored during voltage clamp experiments. We were able to measure changes in fluorescence intensity with most of the dyes and, although the various changes differed in sign and time course, in essentially all cases, the changes depended on membrane potential and not on conductance.

Using polarized light, it was possible to separate two components in the fluorescence change of axons microinjected with 2-p-toluidinyl-napthylene-6-sulfonate (TNS). Both of the changes depended on membrane potential, and thus neither of the TNS binding sites transmit information about the membrane structures responsible for the membrane conductance changes. Our result is not in agreement with the one published recently by Tasaki *et al.* (1972, *J. Membrane Biology,* **8**: 109).

Supported by Public Health Service grant numbers NS 08437 and NS 10489.

Screening pigment migration in the squid. NIGEL W. DAW AND ALAN L. PEARLMAN.

The receptors in the retina of the squid (*Loligo pealei*) contain a screening pigment, located at the junction of the proximal and distal segments of the receptors. Part of this pigment moves to the tips of the receptors in the light, over a period of about 10 min, and in the dark it moves back again, also over a period of about 10 min. Measurements of spectral sensitivity for the screening pigment migration follow the spectral sensitivity of the visual pigment, rhodopsin, rather than the screening pigment, ommin. If more than a certain number of quanta reach the retina, the screening pigment moves out, but does not move back again, even after several hours. The critical number of quanta for this effect is about 1.8×10^{14} quanta cm^{-2}, depending on how much is lost in the media of the eye. This should bleach about 2.7% of the rhodopsin. The quanta can be delivered over any period of time from 6 seconds to 400 minutes, implying that there is some step along the pathway between absorption of light by rhodopsin and migration of ommin which is either irreversible, or reversible with a time constant of more than 7 hours.

This work was supported by NIH research grants EY0053 and EY0621.

A study of the survival of dark mutants of marine luminous bacteria under anaerobic conditions. ANN DIETERICH AND KENNETH NEALSON.

The question of a possible role of the luminescent system of bacteria in their survival under anaerobic conditions was examined through the use of mutants defective in bioluminescence. It has been previously shown that spontaneous dark mutants of the type isolated and described by Keynan do not survive anaerobic conditions which have little or no effect on the wild type. Using these spontaneous dark mutants, we tested many different media, including various carbon and nitrogen sources and various electron acceptors to see if the anaerobic death could be spared in any way. None of the conditions we used were effective in this regard. We also tested twelve dark mutants which were isolated after mutagenesis with MNNG, a chemical mutagen. It had been previously shown that several classes of dark mutants survive as well as the wild type. The twelve which we studied were not of these classes, but were uncharacterized dark strains. Of the twelve strains examined, five survived quite poorly under anaerobic conditions, while the other seven varied from intermediate to as well as the wild type. These twelve mutants were then examined biochemically; by *in vitro* enzyme determinations, by antibody precipitation methods, and by enzyme subunit complementation. They were found to be all similar, being regulation mutants defective in the synthesis of the enzyme luciferase. This is the same property which the spontaneous dark mutants display. We think that it is very likely that the ability to survive under anaerobic conditions is not directly connected to the presence of the luminous system and that the differences seen are more likely due to some frequently associated component of unknown nature.

Effects of Mg^{2+} on skate horizontal cells: evidence for release of transmitter from receptors in darkness. J. E. DOWLING AND H. RIPPS.

The terminals of vertebrate photoreceptors contain the specialized structures that characterize chemical synapses, but the nature of neurotransmission between receptors and their postsynaptic elements (bipolar and horizontal cells) has not yet been determined. Skate horizontal cells exhibit a low resting potential (25–35 mV), always hyperpolarize in response to light, and often show a resistance increase during illumination. Furthermore, sodium aspartate (and other short-chain amino acids) depolarize horizontal cells. These observations suggest that skate photoreceptors continually release a depolarizing transmitter in the dark and that light decreases transmitter flow.

This hypothesis is supported by experiments in which a drop of high Mg^{2+}-Ringer was added to the skate eyecup while recording the electroretinogram and horizontal cell activity. The test solution was prepared by replacing approximately one third of the normal NaCl concentration of elasmobranch Ringer with an equivalent amount of $MgCl_2$ (100 mM). It was assumed that, as at other chemically-mediated synapses, high Mg^{2+} blocks neurotransmitter release from the presynaptic terminal. We found that with high Mg^{2+}, the b-wave of the ERG was entirely abolished, while the a-wave (receptor potential) was only slightly reduced in amplitude. In addition, about 15–25 seconds after applying the test solution, the resting potential of the horizontal cell began to hyperpolarize and there was a corresponding decrease in response amplitude. Within 3 minutes, the resting potential had fallen to about −60 mV and the S-potential was abolished. In two experimental runs, withdrawal of the pipette confirmed the intracellular measurements; *i.e.*, there was a positive shift in potential of 55–60 mV as the electrode left the cell. In another experiment, the membrane potential was monitored for 30 min after applying the Mg^{2+}-Ringer. Following the sequence of events described above, the membrane slowly repolarized to its former level (≈ -30 mV), and light-evoked responses returned to their original amplitudes.

Supported by grants (EY-00824, EY-00285, EY-18766) from the National Eye Institute, and by an award in memory of Harry Groedel from Fight for Sight, Inc., New York.

Oxygen, salinity, pH and temperature variation in the bottom water of Buzzards Bay. EGBERT G. DRISCOLL.

Hydrographic parameters immediately adjacent to the bottom at four stations in northern Buzzards Bay, Massachusetts, have been monitored monthly from October 1971 to August 1972. Water samples were hand pumped into BOD bottles by divers. Collection apparatus consisted of two 30 cm^2 boards separated by a 1 cm distance. Rubber tubing, and an associated rubber bulb with a one-way valve, extend from the center of one board surface to the BOD bottle. The collection apparatus rests on the bottom during pumping and draws water gently from immediately above the sediment-water interface, between the two boards, and into the sample bottle. Samples for dissolved oxygen determination were "fixed" with manganous sulfate and alkaline iodide reagents on the bottom. Oxygen values obtained by this technique differ by as much as 0.15 mg/l from values obtained 0.5 m above the sediment-water interface.

Bottom temperatures in Buzzards Bay range from −0.5° C to 27° C in shallow marginal areas and from 0° C to 22° C in the central area. Dissolved oxygen in the bottom water ranges from 12.2 mg/l in February to 4.3 mg/l in July and is less over mud and muddy sand bottoms throughout most of the year. Dissolved oxygen is also reduced at least 3 mg/l in marginal areas which become frozen over. Salinity ranges from 28‰ to 33‰, and pH from 7.5 to 8.1, throughout the year. The highest June rainfall in forty-eight years resulted in salinity reductions in excess of 2‰ in marginal areas and 1‰ in central portions of the bay.

Annual mean dissolved oxygen and pH values of the bottom water correlate with the mean grain diameter of the substratum, presumably a reflection of the organic content of the sediment.

Long term survival of bioluminescent bacteria in sea water. ANATOL EBERHARD.

In order to investigate their long-term survival under quasi-natural conditions, luminous bacteria were inoculated into sterile sea water and samples were plated out from time to time. Cells of the free-living luminous strain *Photobacterium fischeri* strain MAV survived for two years at a viable cell count of 10^4 to 10^5 per ml. All colonies were luminous but many were small and contained filamentous bacteria. Cells of two strains of luminous bacteria isolated from luminous fish harboring the bacteria as symbionts died out within three weeks. Cells of the free-living strain MAV died out rapidly in 3% sodium chloride but survived as well in artificial as in natural sea water at least for three weeks. That the symbiotic strains do not survive well may explain why such strains are only rarely isolated from sea water even though the luminous fish continuously spill off some of their bacteria. Also, the retention of the luminescent system for two years seems to indicate that bioluminescence has survival value in sea water.

Comparative biochemistry of sea urchin hatching enzymes. BENJAMIN F. EDWARDS AND DENNIS BARRETT.

Reports in the literature assert that (1) hatching enzyme from *Hemicentrotus pulcherrimus* is monodisperse, behaving upon salt fractionation as a single species which is precipitated out of solution by a narrow increment of ammonium sulfate concentration (Yasumasu, 1963); and (2) hatching enzyme from *Strongylocentrotus purpuratus* is heterodisperse, behaving as a single enzyme rather stably associated with heterogeneous contaminants, precipitating from solution over a broad range of ammonium sulfate increments (Barrett, Edwards, Wood and Lane, 1971).

We have explored which of these patterns *Arbacia punctulata* hatching enzyme may follow, for two reasons: if the *Arbacia* enzyme is simple rather than complex, then the species is more favorable for studies of gene expression than is *Strongylocentrotus.* Secondly, a rather tight association between the enzyme and its substrate, the fertilization envelope, would seem to confer a considerable functional advantage: as digestion of the fertilization envelope progresses, enzyme could not diffuse away through holes before the envelope was sufficiently weakened to allow hatching. We wish to test the applicability of this principle.

The result of fractionation of crude hatching enzyme of *Arbacia* by salt precipitation is clear cut. Five successive increments of ammonium sulfate from 2.1 M to 3.9 M (saturation) each bring out additional enzyme activity, and some remains soluble in saturated ammonium sulfate. This result excludes the possibility that the enzyme is a single homogeneous protein species.

Arbacia hatching enzyme, applied in 0.1 M salt to DEAE-cellulose, can be eluted in a single broad peak by slightly raising the salt concentration. This behavior is characteristic of *Strongylocentrotus* enzyme stably bound to contaminant. Recovery of *Arbacia* enzyme is only 50 per cent; a pure fraction may be lost due to instability.

We conclude that by the criteria tested here *Arbacia* hatching enzyme resembles the heterodisperse enzyme of *Strongylocentrotus purpuratus* rather than the simple enzyme of *Hemicentrotus pulcherrimus.*

This investigation was supported by PHS Training Grant No. GMS00265–14 from the National Institute of General Medical Science.

Cation gradient coupled transport of organic substrates—consideration of luminal and plasma sodium, intestine of toadfish Opsanus tau in vivo. A. FARMANFARMAIAN AND GREGG M. FARRELL.

In previous communications we have reported that intestinal absorption of sugars in swimming toadfish can proceed by active transport in the absence of a lumen-to-tissue downhill gradient of Na. These studies have been extended to the transport of L-leucine. It was found that the rates of absorption from salines containing 150 or 0 mM Na were similar at each concentration of L-leucine studied (2, 4, and 8 mM).

The level of correlation between the net transfer of Na and of substrate was investigated by regression analysis. Measured rates from experiments conducted at varied concentrations of substrates made in 150 mM Na saline were used. The correlation coefficient (r) for Na and glucose transfer was 0.08 and for Na and leucine transfer was 0.44, indicating little or no Na interaction with the transfer mechanism of these substrates. In contrast, if it is assumed that Na transfer to and from the lumen is due to solvent drag, the correlation between the measured net transfer and the calculated solvent drag of Na is high ($r = 0.85$). This provides some support for the notion that intestinal absorption of Na is in part due to osmotic flow of solution across the tight junction separating the lumen from the interstitial fluid.

The concentration gradients mentioned above are based on lumen fluid and tissue, rather than intracellular measurements. In a series of *in vivo* experiments the extracellular fluid contribution of plasma and luminal fluids to the intestinal tissue were measured using C^{14}-inulin and H^3-inulin in plasma and lumen fluids respectively. Simultaneously glucose and Na were measured in lumen, tissue, and plasma after steady state distribution of C^{14}-inulin was established in each fish. The data were used to calculate inulin space-corrected intracellular values for Na and glucose. These results further reconfirmed the observation that active absorption of glucose by the intestinal epithelium proceeds in the absence of a lumen-to-cell downhill gradient of Na under *in vivo* conditions.

In part supported by funds from Rutgers University Research Council and Mr. N. Rutgers.

Early receptor potentials and the photochemical cycle of invertebrate photoreceptors. ALAN FEIN.

The early receptor potential (ERP) was measured intracellularly in photoreceptors of the ventral eye of *Limulus* and the lateral ocellus of *Balanus*. The photoreceptors were bathed in isotonic KCl, thereby eliminating the late receptor potential and leaving the isolated ERP. The ERP was elicited by intense flashes from a strobe light.

In *Limulus* a white flash elicits an ERP that is biphasic and mainly hyperpolarizing. The ERP shows only a dependence of amplitude and *not shape* on the spectrum of the flash. If a second flash is presented at an interval of greater than 50 msec the second ERP is also biphasic but is of smaller amplitude than the first ERP. The recovery of ERP amplitude is approximately exponential and has a time constant of 100 msec at 20° C. If a second flash is delivered at an interval of less than 15 msec the second flash elicits an ERP that is mainly depolarizing. The second ERP has a reversed polarity compared to the first ERP. The *shape* of the depolarizing ERP elicited by the second flash is dependent on the spectrum of the flash.

Similar experiments using white flashes were also performed on *Balanus*. The ERP was found to recover with a 40 msec time constant at 24° C. Furthermore, as in *Limulus*, the ERP elicited by the second flash reversed polarity at flash spacings of less than 10 msec.

These results are consistent with the hypothesis that under the conditions of these experiments the photochemical cycle of these receptors is complete in less than one second. Furthermore, because of its reversed polarity and the dependence of its shape on the spectrum of the flash, the ERP elicited by the second flash at an interval of less than 10 msec is interpreted as being produced by a transient photoproduct.

Supported by Special Fellowship 5 FO3 GM42438 from the National Institutes of Health and a Grass Foundation Fellowship.

Plasma proteins in oncotically stressed dogfish. LOUIS FISHMAN, HOPE SCHRIEBER, MURRAY ORATZ, MARCUS ROTHSCHILD AND SIDNEY S. SCHRIEBER.

We were interested to establish if elasmobranchs as exemplified by two species of dogfish-shark, responded to oncotic stress by synthesizing plasma albumin as do most mammals. The mechanism by which elasmobranchs maintain their blood osmolarity slightly hypertonic to sea water is known. By retaining urea and trimethylamine oxide at the gills and by reabsorbing 95% of these metabolites in the kidney little sea water is inbibed and little urine is produced. Elasmobranchs adjust to fresh water or diluted sea water by excreting urea and increased urine flow. Under these conditions of oncotic stress we wished to find out if the liver also responded by synthesizing albumin. Also of interest was the reported observation that *Squalus acanthias* had no blood albumin. To this end citrated plasma was obtained from *Mustellus canis* and *Squalus acanthias* and run on polyacrylamide gel electrophoresis at pH 8.6 with 5 and 7.5% gels. Both species showed broad rapid-moving bands typical of albumin. *Squalus* moved slower than *Mustellus* and both moved slower than bovine serum albumin.

Control samples of blood were taken from sharks of both species. Then they were placed 18.75 or 25% sea water, aerated, and kept at 14–15° C. Additional samples were withdrawn at hourly intervals for as long as it was feasible. Measured were the hematocrit, plasma urea "N," total plasma protein, and albumin. The latter by taking advantage of the solubility of albumins in alcoholic TCA. In *Squalus*, only 1 of 6 fish showed a rise in plasma proteins in response to diluted sea water while, in *Mustellus*, 3 of 6 showed increased albumin synthesis. Further investigation of this response and the characterization of these proteins will be undertaken.

Supported by N. I. H. grants AM-02489 and HE-09562.

The segregation of developmental potential during early cleavage stages in the ctenophore Mnemiopsis leidyi. GARY FREEMAN AND GEO. T. REYNOLDS.

The first differential division during the development of the ctenophore embryo occurs during the formation of the 8-cell stage. Each 4-cell stage blastomere produces one E macromere which subsequently differentiates comb plate cilia and one M macromere which differentiates light producing cells. The time at which this segregation of developmental potential occurs during development has been studied here by removing defined cytoplasmic regions from blastomeres at the 2- and 4-cell stages and studying the ability of the nucleated portion of the blastomere to undergo subsequent differentiation.

At the 2-cell stage nucleated fragments were produced that would normally form either the E or the M macromeres at the 8-cell stage. These fragments were allowed to cleave twice; after each of these cleavages the blastomeres were separated. The two daughter cells which were derived from the last cleavage were raised as isolates together and subsequently scored for comb plate cilia and light producing cells. The majority of these isolate pairs formed one comb plate cilia and one light producing isolate, indicating that a segregation of developmental potential had not taken place.

At the 4-cell stage nucleated fragments were also produced that would normally form either the E or the M macromeres at the 8-cell stage. These fragments were allowed to cleave once, then the blastomeres were separated and raised as isolate pairs. The majority of the fragments which were derived from the part of the 4-cell stage blastomere that would normally form the E macromere formed one comb plate cilia and one light producing isolate. All of the fragments derived from the portion of the 4-cell stage blastomere that would normally form the M macromere produced pairs of isolates that only differentiated light producing cells. This result indicates that at the 4-cell stage the segregation of developmental potential has begun to occur in one part of the blastomere, but not in another part of the same blastomere.

This research was supported by AEC Contract AT (30–1)–3406 to G. T. R.

Unusual aspects of chromosome movement and phragmoplast formation in Tilia americana. John W. Fuseler.

Maturing seeds, approximately 6.5 mm long, of a basswood, *Tilia americana*, contain large, clear endosperm cells. Their mitosis is exceptionally suited for polarized light microscope studies, although only available three days per year. Cells are spread with FC-47 fluorocarbon oil. On the metaphase plate the 124 chromosomes form a single straight row. Anaphase onset is characterized by an abrupt separation of all the chromosomes. The chromosomes move synchronously as two parallel rows until they bow *away* from the poles just before phragmoplast formation in mid-anaphase. Such precocious phragmoplast formation has not been previously reported. Through most of anaphase the velocity of chromosomes near the spindle axis remains constant and is unaffected by phragmoplast formation or bowing of the chromosome rows at any temperature. Anaphase chromosome velocity increases exponentially with increasing temperatures between 10° and 25° C. Outside this range anaphase movement ceases. The anaphase half-spindle birefringence (BR) decay rate also increases as an exponential function of temperature. The linear positive relationship between BR decay rate and chromosome velocity indicates that the greater the rate of spindle depolymerization, the greater the rate of chromosome movement. This is consistent with the dynamic equilibrium model of chromosome movement, but is not readily explained by the sliding filament model. That the dynamic equilibrium model can be applied to plant mitosis is further substantiated by analysis of metaphase equilibrium BR at various temperatures. Using Inoué's formulation, a linear van't Hoff plot gave a change of enthalpy, entropy, and free energy of 33.8 kcal, 123 eu, and 0.2 kcal per mole, respectively. These high positive values of enthalpy and entropy suggest that in plant spindles also an entropically or hydrophobic bond driven equilibrium exists between monomer and birefringent polymer (microtubules).

Supported in part by Training grant 5T01HD00030, and grants CA10171 from NIH and GB31739X from NSF.

Identification of some membrane protein subunits obtained from the squid giant axon. H. Gainer, E. Carbone, I. Singer, K. Sisco and I. Tasaki.

An enzymatic iodination procedure was used to probe for accessible proteins in the outer and inner surface layers of the squid giant axon. The reaction mixture contained 60 μg lactoperoxidase, 12 μg glucose oxidase, 100 μg glucose and 20 μCi carrier-free NaI^{125}, dissolved in 1.5 ml of either filtered sea water (external medium) or internal perfusion fluid (400 mM potassium phosphate, 4% glycerol, pH 7.3) for corresponding surface layer iodination. Axons were exposed to the external reaction mixture for 10 min, and then the enzymes and unreacted I^{125} were washed off with large volumes of pure sea water. Other axons were perfused with the internal reaction mixture for 10 min after removal of most of the axoplasm; these axons were then extensively washed internally with perfusion fluid to remove the reaction mixture. The conduction velocity of the action potential decreased by one half during the latter procedure. The radioiodinated proteins were solubilized in 1% SDS- 5% β-mercaptoethanol, and were analyzed by disc electrophoresis in SDS.

The iodinated proteins obtained from the external surface layer had a wide distribution of molecular weights, with a dominance at the higher molecular weights (*i.e.*, >200,000 daltons). In contrast, the labelling pattern of the proteins obtained from the internal surface layer was much simpler, with only two major peaks of radioactivity (at about 68,000 and 12,000 daltons), with dominance of the lower molecular weight peak. Depolarization of the axon with high concentrations of externally applied potassium ions appeared to decrease the efficacy of iodination of the proteins obtained from the internal surface layer. The proteins labelled by internal perfusion are not believed to derive from non-specific labelling of any remaining axoplasm, since electrophoresis of isolated axoplasm proteins gave a completely different molecular weight profile, with at least 16 major bands and dominance at 170,000, 40–59,000, and 19–27,000 daltons.

Dr. I. Singer is an Established Investigator of the American Heart Association (69–106), and is supported, in part, by a grant from the U. S. P. H. S. (5–R01–14012–02).

The isolation and uptake of vitellogenin by spider crab oocytes in vitro. L. R. GANION, R. E. BAST AND R. A. WALLACE.

Serological and electrophoretic studies suggested that an orange, female-specific protein (vitellogenin) occurs in the serum of *Libinia emarginata* during vitellogenesis. Such serum was equilibrated against 50 mM Tris-citrate-1 mM EDTA (pH 7.0) and applied to a DEAE-cellulose column. A non-absorbing, orange fraction (5–10% of the applied protein) was collected, dialyzed against 50 mM sodium acetate (pH 5.0) and applied to a CM-cellulose column; a single peak was eluted with a salt gradient. These procedures permitted a considerable purification of vitellogenin, but electrophoretic analysis on polyacrylamide gels still indicated the presence of several protein contaminants.

A culture medium was then devised in order to study the incorporation of vitellogenin into developing oocytes. Labeled vitellogenin (1.6×10^6 dpm/mg) was prepared by reductive alkylation with ^{3}H-formaldehyde, added to male serum and dialyzed against a salt solution [0.78 M NaCl, 15.7 mM KCl, 22.4 mM $CaCl_2 \cdot 2H_2O$, 44.3 mM $MgCl_2 \cdot 6H_2O$, and 10 mM HEPES buffer (pH 7.5)/liter]. The dialyzed protein was then diluted 1:1 with double strength Medium-199 containing 2 mM HEPES (pH 7.2) (final vitellogenin concentration = 0.42 mg/ml; final serum concentration = 17%). Vitellogenic ovarian fragments and isolated oocytes (0.45 mm diameter) were then incubated for up to 9 hrs in this medium plus antibiotics, both in the presence and absence of ^{14}C-leucine. The incorporation of leucine into TCA-insoluble material proceeded with linear kinetics, indicating oocyte viability. The uptake of ^{3}H-vitellogenin by isolated oocytes was also linear throughout the experiment (6.4 ng/oocyte/hr), whereas a linear uptake by ovarian fragments proceeded for about 6 hr and at a reduced rate. After incubation, doubly labeled ovarian fragments were washed, homogenized together with unlabeled ovary, and centrifuged at 27,000 × *g* for 30 min. Dialysis of the orange supernatant against 50 mM sodium acetate (pH 5.0) yielded an insoluble and soluble fraction. The latter eluted as a single peak (lipovitellin) on a CM-cellulose column. This material was exclusively labeled with tritium whereas the centrifugal pellet and pH 5.0 precipitate were labeled with ^{14}C. These data indicate that isolated, developing oocytes can both synthesize endogenous (non-lipovitellin) protein and sequester a lipovitellin precursor from the medium.

This study was performed in the Fertilization and Gamete Physiology Training Program at the MBL (NIH grant 5-T01-HD00026–11).

Isolation and host range studies of marine bacteriophage. MARY LOU GILL AND KENNETH NEALSON.

Viruses which attack marine bacteria represent a poorly documented group of bacterial predators. We tested several techniques for studying the biology and ecology of marine bacteriophage. The technique of simultaneous enrichment with several different hosts was found to be quite successful providing that the host bacteria had similar growth rates. When strains with widely different growth rates were included in the same enrichment flask, no phage were ever found which were active against the slow growers. Slow growers by themselves gave positive results. It was found that aeration of the enrichment cultures was important for good yields of phage; when this was omitted, few or no phage could be found. The standard method was to inoculate one liter of non-sterile sea water with equal amounts (10^9) of up to eight strains of bacteria, 5 g of tryptone, 5 g of yeast extract, and 3 ml of glycerol, and allow this to grow with vigorous bubbling of air for 24 hours. For identification of the phage, we attempted to use a spot test, putting a drop of the extract to be tested on a lawn of bacteria. From 30 to 70 per cent of the phage known to be present were missed by this method; standard plaque methods with soft agar overlay were much better. Using Eel Pond water as a source of phage, we attempted enrichments with three groups of bacteria; (1) Eel Pond isolates, (2) other Woods Hole isolates, and (3) bacteria from diverse sources. Host range studies done with the various phage indicate that they may be of some value in the systematic identification of marine bacteria, or for the study of the relationships between bacteriophage and their microbial hosts.

Potassium dependence of the hyperpolarizing receptor potential of the scallop photoreceptor. A. L. F. GORMAN AND JOHN S. MCREYNOLDS.

Not only vertebrate rods and cones, but also some types of invertebrate photoreceptors respond to light with a hyperpolarizing receptor potential. Although the vertebrate photoreceptor response, like other receptor potentials, appears to be due mainly to a change in Na^+ conductance, nothing is known about the ions involved in the hyperpolarizing responses of these invertebrate photoreceptors. We have previously shown that the hyperpolarizing receptor potential of the distal photoreceptor cells of the mollusc, *Pecten irradians*, is associated with an increase in membrane conductance, with a reversal potential near -80 mV. We have now developed a technique for changing the ionic composition of the fluid surrounding these small cells during continuous intracellular recording of the changes in membrane potential and response to light. Complete replacement of extracellular Cl^- ions with impermeant anions had negligible effect on either the resting potential or the response to light. Conversely, the potential reached by the response to light varied inversely with log external K^+ concentration over a wide range. The resting, of dark, potential also varied inversely with log external K^+ concentration, but the slope of this relationship was much less steep. Replacement of external Na^+ with impermeant cations resulted in hyperpolarization of the resting potential and a decrease in membrane conductance. Our interpretation is that the membrane is kept at a low value in the dark by a high Na^+ permeability whereas the receptor potential is due primarily to an increase in permeability to potassium ions.

Release of protease activity from sea urchin eggs upon fertilization. ALBERT GROSSMAN, WALTER TROLL, MILTON LEVY, LOUIS FISHMAN, GERALD WEISSMANN, JEFFREY LASKIN AND ROY SOBERMAN.

Sea urchin eggs contain a protease capable of hydrolyzing tosylarginine methyl ester (TAME) and protamine. Using TAME-H^3 as substrate for this protease, a continuous assay (within a scintillation vial) was employed which could detect 0.2 nanogram of trypsin-like enzymes. Within 5 min after fertilization the TAMEase activity of early sea urchin embryos decreased about 30%. Approximately 50% of the initial TAMEase activity was detectable after 15 min. No further change in TAMEase activity was noted between 15 and 30 min after fertilization. The observed decrease in TAMEase activity could be stoichiometrically accounted for in the sea water in which the eggs were fertilized.

The TAMEase activity released into the sea water was almost entirely lost after Millipore filtration (cellulose nitrate filters having a pore size of about 0.45 μ). However, no loss of activity was noted after centrifugation at 100,000 $\times g$ for 1 hour. These results suggest that the enzyme may be bound in a seemingly soluble complex which can be readily adsorbed on to cellulose nitrate filters. Disc electrophoresis of sea water samples in which sea urchin eggs were fertilized indicated a single fast moving band that was shown to contain TAMEase activity. This would indicate that any association with a larger substance can be readily dissociated in an electric field. Whether the association of TAMEase activity and a macromolecule or particle exist within the unfertilized egg remains to be determined.

This investigation was supported by grant #GM-13728 from the USPHS.

Lens epithelial cell surfaces in marine fish. C. V. HARDING, D. HARDING, V. PETERS, T. KUWABARA, J. REDDAN, N. UNAKAR, M. BAGCHI, T. SCHNUR AND S. GORDON.

Observations on the normal lens epithelial cell surfaces with the scanning electron microscope (SEM) have been carried out as part of an overall study on wound healing. The present report consists of observations on the normal lens epithelium of toadfish and dogfish.

The apical cell surfaces were studied after the capsule (basement membrane) with adhering epithelial cells was mechanically separated from the fiber cells in fixed lenses. The *basal* cell surfaces were examined after the capsule, or most of the capsule, was removed by collagenase treatment. It proved possible to obtain a clean separation of fiber cells and epithelial cells, thus enabling visualization of the apical cell surfaces with the SEM. Preparations which had been scraped revealed cell fragments and the inner capsular surface. Clean whole cell nuclei were clearly evident. A hexagonal pattern appeared on portions of the inner capsular surface. The size of the hexagons corresponds to that of the epithelial cells. It is suggested that the hexagons define regions of maximum adhesion between cell and capsule, or possibly regions at which the cell is most likely to fragment. Examination of the lens surface after collagenase treatment also revealed in some areas a hexagonal pattern. The size of these hexagons corresponds to that seen on the inner capsular surface, and it is believed that the two hexagonal patterns represent one pattern as seen from the two sides. We suggest that the lens provides a useful model system for studies on the relationship between epithelium and basement membrane.

These experiments were supported in part by U. S. A. E. C. Contract AT(11–1)1427 and grant EY 00362 from the National Eye Institute, N. I. H.

Multivalent and univalent concanavalin A as probes for studying sperm-egg interactions. CRAIG W. S. HOWE AND CHARLES B. METZ.

Concanavalin A (Con A), a lectin which binds macromolecules containing a-D-mannopyranosyl or a-D-glucopyranosyl residues as determinant sugars, inhibits fertilization in the sea urchin *Arbacia punctulata.* The inhibition is concentration dependent (50% inhibition at 100 μg/ml) and is specifically inhibited with methyl a-D-glucopyranoside. Con A does not agglutinate gametes or produce any observable morphological changes in these cells.

Con A does not affect the fertilizing capacity of sperm. However, it combines with sperm as shown by absorption of Con A and by anti-Con A agglutination of Con A treated sperm. Likewise, Con A does not affect the sperm agglutinating action of *Arbacia* egg jelly solution (fertilizin). The vitelline membrane of the egg is a site of Con A action. Fluorescein conjugated Con A binds to the vitelline membrane as revealed by a uniform distribution of egg surface fluorescence. In addition, dejellied eggs attach to Con A coated Sephadex particles and rabbit erythrocytes. Con A agglutinates demembranated eggs (trypsin treated), indicating further receptors presumably beneath the vitelline membrane. Finally, trypsin treatment restores fertilizability to dejellied, Con A treated eggs.

The effects of local concentrations of Con A were evaluated by exposing eggs to Con A coated Sephadex particles. Eggs bound to a single particle did not fertilize; unbound eggs elevated fertilization membranes and cleaved. Con A was present only on the bead surface because the supernatant failed to agglutinate erythrocytes.

In immunoelectrophoretic and immunodiffusion tests, Con A precipitated certain components of an *Arbacia* egg homogenate, some of which were also precipitated by anti-egg antibodies.

Papain digestion of the Con A dimer produced fragments that bound to, but no longer agglutinated, erythrocytes. The univalent fragments alone did not inhibit fertilization, but when eggs that had bound fragments were treated with anti-Con A no fertilization membranes elevated. Thus, Con A appears to inhibit fertilization by a cross-linking of moieties containing the specific sugars rather than by masking of receptors.

This study was performed in the Fertilization and Gamete Physiology Training Program at the MBL (NIH grant 5-T01-HD00026–11).

Arbacia *spermatozoa: plasma membrane* "in rigor." SADAYUKI INOUE.

The outer surface of plasma membranes from *Arbacia* sperm and eggs has an assembly of fine filaments occasionally in ordered array. Whether these membrane components are similar to contractile proteins is under debate. The rigid actin-myosin complex of rigor is established. When sperm membranes are heat-immobilized *in vitro,* the unaltered ultrastructure of the surface layer may resemble muscle rigor, if contractile proteins are present.

Dry sperm (10 ml) from *Arbacia punctulata* were diluted to 500 ml with filtered sea water in which penicillin (100 units/ml) and streptomycin (0.1 mg/ml) were added in order to prevent bacterial contamination. Aliquots of the suspension were incubated at 30° C for ten intervals from 2 hrs to 37 hrs. Incubated sperm were collected on Millipore filters, washed 3 times either with sea water, or with distilled water, and were freeze dried. Surface replicas were made for electron microscopy.

The medium varied from pH 7.8 to pH 7.2 during 37 hr of incubation. Repeatedly, after 10 hr, the cells became resistant to H_2O and, concomitantly, an ordered structure appeared on the surface of the sperm membrane. This structure resembled a parallel arrangement of "helical" (zig-zag) filaments averaging 30 Å in diameter. The period of overlap between filaments is 70 Å. The filamentous layer appears as a crossed-grid network with spacing of 50 Å in both directions and a cross angle of 75–80°. It is postulated that chemical bonds or bridges between neighbouring filaments are formed or altered at their overlapping positions in response to physiological conditions of living spermatozoa.

Stiffness measurement of skinned muscle fibers at low frequency. MASATAKA KAWAI.

Stiffness change of skinned crayfish muscle fibers was studied at various degrees of activation in the presence or absence of Ca^{++} ions. The length displacements (0.2–0.5% fiber length) were either of ramp or sinusoidal (5–10 Hz) form produced by a speaker servo system. The stiffness was not a function of frequency in the range of 1–20 Hz. The sarcomere length was measured by laser diffraction technique and was initially adjusted to 8.0 microns. The stiffness is minimal in the resting skinned fiber bathed in 200 mM K-propionate, 3 mM EGTA, 1.45 mM ATP, 1.45 mM Mg (pMgATP = 3.0), 5 mM tris-maleate (pH 7.0). Upon removal of MgATP (rigor) the stiffness increases. Reintroduction of micromolar quantities of MgATP produces a progressive increase in tension and stiffness, both reaching maximum at about 3 μM MgATP. Further increasing the concentration of MgATP (up to 1 mM) proportionally reduces both tension and stiffness. In the presence of Ca^{++} (pCa = 5.0) at pMgATP = 3.0, tension can be increased by approximately two-fold; however, concomitant increase in stiffness is only about 20%. Elastic moduli for resting, rigor, MgATP (3 μM)-activated tension, and Ca-tension are about 0, 30, 65, 80 kg/cm^2, respectively. In addition incremental stiffness of Ca-activated fibers was measured at various sarcomere lengths. Since the stiffness of the activated muscle is proportional to the degree of overlap between the thick and thin filaments, one can conclude that the stiffness is a measure of actin-myosin interaction. The theoretical implication of the nonequivalence of tension and stiffness will be discussed.

Supported in part by funds from the Muscular Dystrophy Associations of America, Inc., by Public Health Service grants NS 03728 and NS 05910, and training grant NS 05328, and from the National Science Foundation (GB 6988X).

Glycoprotein and glycolipid mannosyl transferases of dogfish retina. EDWARD L. KEAN, WILLIAM E. BRUNER AND PATRICIA C. SHERWOOD.

Although mannose-containing lipids have not been isolated from animal tissues, the biosynthesis of such compounds has been observed. It has been suggested that these compounds may function as precursors to mannose in glycoproteins. We have observed the presence of enzymes in the retina of the dogfish that catalyze the incorporation of mannose from GDP-mannose-^{14}C into endogenous lipid and glycoprotein acceptors. Retinas were homogenized in the light with 0.25 M sucrose (3 sucrose:1 tissue, v/w), and then dialyzed against 0.1 M KCl.

Incubations were performed in the presence of $MnCl_2$ (3.3 mM); TES buffer (0.1 M, pH 7.0); GDP-mannose-^{14}C (1.74 μM, 154 μCi/μmole) and enzyme (up to 1.4 mg protein) for 5 min at 37°. The reaction was stopped by the addition of cold 6% trichloroacetic acid-0.5% phosphotungstic acid. After centrifugation, the lipid was extracted from the pellet with chloroform-methanol (2:1). Radioactivity was determined in the washed lipid extract and in the residual pellet. The following activities (cpm/g retina/min) were observed: [mean ± SE (number of determinations)]: lipid, 10,100 ± 995 (9); glycoprotein, 4110 ± 394 (9). Most of the enzymatic activity was associated with the pellets sedimenting at $480 \times g$ ("nuclei" + debris) and at $105,000 \times g$ ("microsomes"). The time courses of reactions were consistent with the possibility that the lipid product served as a precursor for the radioactivity incorporated into the protein.

Supported in part by USPHS Research Grant EY 00393.

What you can do with amino acid analyses. DONALD M. KIRSCHENBAUM.

Amino acid analyses, the "elemental analyses" of proteins, are useful for assessing the compositional relatedness among proteins, and for estimating the size, shape, hydration, electrophoretic mobility, net charge, steric factors, specific volume, refractive index, molar absorptivity, and $\mathbf{A}_{1\,\mathrm{cm}}^{1\%}$ of individual proteins. The mean residue rotation, sedimentation coefficient, intrinsic viscosity, and root-mean-square end-to-end distance of 6 M guanidine HCl denatured proteins can also be estimated. Certain structural characteristics of proteins, such as the content of β-structure, α-helix, and random coil can be estimated semi-quantitatively. Phylogenetic relationships, base compostion of genes, and peptide bitterness can be determined. In the field of collagen chemistry the content of total imino acids can be related to thermal stability [shrinkage and melting temperature], thermal transition temperature [denaturation], and body temperature of modern and ancient animals. Amino acid analyses provide for prediction, estimation, and speculation concerning structural, phylogenetic, and perhaps functional characteristics of proteins using techniques reported in the literature. In addition, the "purity" of a protein preparation may be monitored by comparing the values of certain calculated parameters with experimentally determined values.

Facilitation and depression of transmitter release in the squid giant synapse. E. M. LANDAU AND K. KUSANO.

The processes of facilitation and depression of transmitter release in the squid giant synapse have been examined with intracellular microelectrodes, placed pre-synaptically for stimulating and recording and post-synaptically for recording. Nerve conduction was blocked with 2×10^{-7} M TTX. It was found that when the synapse was stimulated repetitively ($2 \sim 15$ stimuli at 50 sec^{-1}), both synaptic depression and facilitation occurred. Thus, the consecutive post-synaptic potentials (PSP's) in a tetanic train became progressively smaller, indicating depression of transmitter release. However, single test pulses, applied at 0.1 to 1 sec after the end of the tetanus, consistently produced PSP's which were smaller than the smallest PSP in the tetanic train. We take this as evidence for a process of synaptic facilitation. The time constant of this facilitation was found to be less than 100 msec. The synapse recovered from the depression in an exponential manner with a mean time constant of 5.1 sec (range 3.2 to 6.5 sec in 4 experiments). This time constant was independent of the size of the PSP.

By employing a non-linear model for depression (*cf.* Betz, 1970, *J. Physiol.*, **206**: 629) we were able to evaluate "p"–the probability of transmitter release. The values obtained were less than 0.12, which is in agreement with similar estimates from other preparations. We examined the relationship between "p" and the amplitude of the PSP, the latter being varied by changing the bathing calcium concentration. This relationship was found to be linear indicating that calcium did not affect "n," the store of immediately releasable quanta. On the other hand, when the PSP's were varied by changing the pre-synaptic peak depolarization the relationship between "p" and the PSP amplitude was not linear, but became less steep as the PSP's increased in size. This may be taken to indicate that when a large depolarizing pulse is applied to the pre-synaptic membrane not only "p," but also "n" increases to some extent.

Supported by NINDS grant (NS 09618) and UCP grant (SG-28-72).

The effect of temperature on the influx of sodium ions associated with nerve impulses in the perfused squid giant axon. DAVID LANDOWNE AND LAWRENCE B. COHEN.

The influx of sodium ions associated with nerve impulses was studied by extruding the axoplasm of a squid giant axon and then perfusing the axon with a KF solution. The preparation was bathed in sea water made radioactive with Na-22 or Na-24. The influx of sodium was measured by collecting the perfusion fluid and assaying it for radioactivity. Stimulation at thirty impulses per second produced a large increase in the influx. The nerve was cooled from room temperature (18–25° C) to 2–6° C. Cooling slightly decreased the resting influx, with a Q_{10} of 1.4 and slightly increased the extra influx associated with impulses. The Q_{10} for six bracketed measurements of the extra influx was $1/1.56 \pm 0.06$ (standard error).

Essentially the same results have been found by one of us (D. L.) for intact axons. Thus, the extra sodium influx has a Q_{10} of 1/1.43 and the extra efflux, 1/1.3. The consistency of the results of these three quite different determinations indicates that this is not a simple measurement artifact.

The significance of these findings is that they are quite different than one would expect from the classical model. Hodgkin and Huxley predicted the extra influx of sodium would have a high temperature dependence, with a Q_{10} of 1/3.0. Our data suggests that it will be necessary to seek a new explanation for the ionic currents associated with nerve impulses.

Supported by Public Health Service grant number NS08437.

Sponge cell adhesion: Velocity sedimentation separation and aggregative specificity of discrete cell types. ARDEAN G. LEITH AND MALCOLM S. STEINBERG.

Microciona prolifera dissociated in calcium-magnesium free artificial sea water (CMF) by Humphrey's procedure yields a heterogeneous cell population. Certain cells (the so-called archeocytes or grey cells, called NBS+ here) can be vitally stained with nile blue sulfate. The remaining NBS− cells are of two major types: orange-vacuoled, nucleolated cells and clear cells containing red granules (H. V. Wilson's "collar cells"). The aggregates formed in the presence of "aggregation factor" are of two types: large aggregates containing both NBS+ and NBS− cells with the NBS+ cells located in tight clumps within the NBS− mass; and small, loose aggregates containing predominantly NBS− cells.

The three cell types are separable by velocity sedimentation. Stained CMF-dissociated cell suspensions were layered in a cylindrical chamber between a layer of CMF and a 5% step gradient of sucrose in CMF and were allowed to sediment for 3–5 hours. The different cell types sediment at different velocities, with the NBS+ cells taking up the bottom position, nucleolated cells the middle, and granule cells the upper position.

"Factor" promotes the aggregation of all three cell types; separated NBS+ cells form compact rounded aggregates while cells of the other two fractions form loose, flaky aggregates. Combination of NBS− and NBS+ cells plus factor results in the formation of compound aggregates with NBS+ clumps enclosed by the NBS− cells.

When *mechanically* dissociated cells are substituted for CMF dissociated cells, many of the NBS+ cells are observed on the *surface* of aggregates, upon which they subsequently spread.

The differences in aggregative specificity of NBS+ and NBS− cells might be explained by invoking differences in the number of "factor" linkages possible for the various cell types; or alternatively, "factor" preparations may be heterogeneous, containing a variety of "factors" differentially bound by the various cell types.

Aided by NSF grant GB-5759X and ACS grant BC-52B.

Binding of indicator anions by protamine and its use in a sensitive assay of protease. MILTON LEVY AND WALTER TROLL.

To examine the numerous postulates involving proteases in physiological situations requires a sensitive and convenient assay. A protein (rather than synthetic esters or amides) substrate should lend assurance to findings by the method. We have developed such an assay based upon the fact that anions of sulfonphthaleins combine with protamine but not with its hydrolysis products. The color of cresol red for instance is much redder in the presence of protamine than the pH of the medium calls for. By working on the acid side of its pK and with limited quantities of buffer salts the color change can be used to follow directly the hydrolysis of the protamine with a spectrophotometer. The method in this form is very sensitive to salts and the concentration of substrate is limited. A more versatile but discontinuous method takes advantage of the much greater sensitivity of the color at lower levels of protamine. The enzyme is added to a buffered, pH 8.0, protamine solution and samples are taken into bromphenol blue solutions containing sufficient formic acid to reach pH 3.5 where the action will stop. The color is measured and its rate of change compared with trypsin standards.

The method has been used to demonstrate the release of a protease at fertilization in sea urchin eggs. It is conveniently used with 0.2 micrograms of trypsin.

Characterization of bioluminescent bacteria by studies of their inducers of luciferase synthesis. JAMES MAGNER, ANATOL EBERHARD AND KENNETH NEALSON.

In 1970, Mitchell and Hastings proposed on the basis of the decay kinetics of luciferases isolated from different strains of bacteria, that the luminous bacteria consist of two distinct groups, the free living and the symbiotic types. Eberhard, working with one strain of each group has shown that the luminous bacteria produce a small molecule which accumulates in the medium and results in induction of the synthesis of the luminescent system, and that the inducers produced by the two different strains did not cross react with respect to enzyme induction. We chose to examine this property of inducer cross reaction in order to determine whether it might be used as a tool for which to examine the relatedness of luminous bacteria. To do this, we chose two strains, one a local Woods Hole free living isolate, and one a purported freshwater luminous strain (*Vibrio albensis*) obtained from Scotland. The latter was of particular interest, as luminosity is thought to be a property which is confined to marine bacteria. With regard to this problem, it was found that *V. albensis* would grow on a medium with no sodium chloride, but that it's optimum of growth occurred at 3% salt, characteristic of other marine forms. With regard to the inducers of the strains, it was found that both the freshwater isolate from Woods Hole, and *V. albensis* produced an inducer which cross reacted unambiguously with the free living type (MAV). Since the inducers from the two types of bacteria differ in many other respects, it may be quite reasonable that they have evolved to be useful in separate niches, and that characterization of the bacteria by the type of inducer would be a good systematic approach to understanding their ecology.

The effect of electrical stimulation on the action of sulfhydryl reagents in the giant axon of squid. JUDITH K. MARQUIS AND HENRY G. MAUTNER.

Studies with sulfhydryl reagents on squid giant axons by Hillman and Mautner in 1968 demonstrated that conduction block by PCMB and NEM was potentiated by repeated, brief stimulation of the partially-blocked nerve fiber. We have been able to quantitate these studies and have shown, with NEM, a relationship between dose of SH inhibitor and number of stimuli required to produce inexcitability. The compounds were applied externally to produce 10–20% decrease of AP amplitude. The fiber was subsequently stimulated at threshold 1–6 times/sec which produced total inexcitability in about 30–90 sec. It is necessary to partially block conduction in order to observe block by stimulation. PCMB, mercurochrome, and fluorescein mercuric acetate (5×10^{-6} M) produce conduction block reversible with 10 mM beta-mercaptoethanol and exhibit the "stimulation effect." NEM, DTNB, and 2-dimethylaminoethyl selenolbenzoate also exhibit the "stimulation effect," but conduction block is irreversible. In a series of three potent local anesthetics—procaine, 2-diethylaminoethyl thiolbenzoate, 2-dimethylaminoethyl selenolbenzoate—only the selenium analogue reacts with SH groups, and it is the only one which shows a "stimulation effect." Possible interpretations of this effect include: increased permeability, unmasking of buried SH groups in the membrane, and electrolytic reduction of disulfides producing additional SH groups. We are unable at this point to eliminate any of these possibilities. There is evidence against phospholipid interactions from the experiments with local anesthetics and from an experiment in which partial procaine block is followed by application of NEM. NEM must inhibit an additional 10–20% before the stimulation effect is observed. This supports the idea that the two reagents are acting at separate sites. Kosower and Werman have suggested that the "thiol status" of neurones is involved in their functions. Our data are compatible with that postulate.

This work was supported by grants from the National Institute of Neurological Diseases and Stroke (NS-09608) and the National Science Foundation (GB-31845).

Spatial and temporal genetic variation in Mytilus edulis: *natural selection and larval dispersal.* ROGER MILKMAN, RODNEY ZEITLER AND JOHN F. BOYER.

Mussel populations in an estuarine complex in Sandwich, Massachusetts, show progressive differences in *LAP* allele frequencies between the mouth and a site 800 m upstream. For successive size classes, *p*, the frequency of the slow allele, is 0.36, 0.22, 0.18, 0.52, and 0.52 at the mouth, and 0.37, 0.18, 0.24, 0.30, and 0.17 upstream. We attribute the differences to natural selection, and we attribute the fluctuations within populations to fluctuations in the proportionate contributions from Northern populations (where *p* is around 0.10) and Southern populations (where *p* is close to 0.55). Such fluctuations have now been observed in several locations in and near the Cape Cod Canal but not in numerous populations to the southwest.

Within the Sandwich estuary, intermediate locations show intermediate frequencies, and a similar pattern is seen in a nearby estuary at Scorton. Vineyard Haven Harbor, accessible to larvae from both Northern and Southern populations, also reveals an inward size-dependent decline in *p* to 0.29 at the Lagoon Pond entrance in larger mussels, while two populations just outside the harbor have 0.52 and 0.50, respectively.

Because of the remarkable uniformity of the Southern populations (with the striking exception of those in Long Island Sound studied extensively by R. Koehn) over a great diversity of habitats, it is evident that selection is not operating directly on the *LAP* locus, but rather on the associated genotypes of Northern and Southern populations, respectively, whose proportionate contribution to a mixed population can be estimated by *p*.

Since the differences among mussels in a given area such as an estuary seem to increase sharply with size, differences in settling sites chosen by contemporaneous Northern and Southern larvae are not indicated.

Supported by USPHS grant GM 18967 to R. M. and a research fellowship to J. F. B. under the Program in the Biological Sciences at the University of Iowa funded by the National Science Foundation.

Spectral and kinetic characteristics of bioluminescence in Pelagia noctiluca *and other coelenterates.* JAMES G. MORIN AND GEORGE T. REYNOLDS.

Bioluminescence from the scyphomedusan *Pelagia noctiluca,* like all other light producing coelenterates that have been examined, involves the specific activation of a photon emitting photoprotein molecule by calcium. However, the light production from *Pelagia* differs from the other coelenterates in several ways. First, the *in vivo* emission spectrum of *Pelagia* is broad and unstructured with the peak at about 475 nm. In this regard it has a spectral shape similar to that of *Mnemiopsis leidyi* (λ_{max} 480 nm) and *Parazoanthus lucificum* (λ_{max} 520 nm), but is quite distinct from the hydroids (*Obelia geniculata* and *Clytia edwardsi*) and the pennatulids (*Renilla köllikeri, Ptilosarcus guerneyi* and *Acanthoptilum gracile*) which have a structured emission with a narrow peak at about 508 nm. All of these spectra were made by the method of Reynolds *et al.* described elsewhere in these meetings.

Pelagia has been reported and confirmed here to have an extracellular luminescence; this is unlike all of the above species. Consistent with this observation is the remarkable similarity of the kinetics of the decay of the light between the *in vivo* electrically induced flash (rate constant $[k] \cong 2.2\ s^{-1}$) and the *in vitro* flash from the calcium triggered isolated photoprotein ($k \cong 2.0\ s^{-1}$). In all of the other luminescent coelenterates studied there is an approximately ten fold difference between the *in vivo* and *in vitro* reactions and this probably indicates that calcium is actively removed from their *in vivo* intracellular reactions. Some examples of these *in vivo/in vitro* rate constants (in s^{-1}) are: 54/3.4 for *O. geniculata,* 8/0.16 for *R. köllikeri,* and 24/3.5 for *M. leidyi.* In *Pelagia* the *in vivo* rise time (*ca.* 100 ms) and one half decay time (*ca.* 330 ms) are much slower than any of the *in vivo* flashes observed in other coelenterates.

Finally, *Pelagia* has proven unique among the coelenterates in its *in vivo* response to repetitive stimuli. A single flash of light occurs after the first stimulus of a train, this and successive flashes are localized to the region of stimulation, and the series of flashes show an approximate exponential decrease in maximum light intensity within the train. On the other hand, other luminescent coelenterates do not respond until at least the second or third stimulus, most show some form of a propagated flash, and display a facilitation of the maximum light intensity from flash to flash. The above experiments were done at 19 ± 1° C.

Polarization sensitivity ratios of retinular cells in the crabs Carcinus *and* Callinectes *under conditions of selective adaptation and dim stimuli.* MICHAEL I. MOTE.

Studies of the mechanism of polarized light sensitivity in decapod crustaceans has led to a curious paradox. Measurements of dichroism in isolated rhabdoms using microspectrophotometry have revealed an absorption ratio of 2:1. This is consistent with the model of random orientation of visual pigment molecules in the plasma membrane. Measurements of polarization sensitivity with intracellular microelectrodes, on the other hand, yield average ratios of between 4 and 6 to one. Two general hypothesis exist to explain this phenomenon. First, that the visual pigment is not oriented randomly in the plasma membrane and that the dichroic ratio is more than 2 in the *intact* cell. Secondly, the dichroic ratio is 2, but that this value is enhanced through interactions between cells whose maximum sensitivities have orthogonal planes. The experiments described test these hypotheses. Retinular cell responses to polarized light stimuli were recorded with standard microelectrode techniques. Polarization sensitivity ratios ranged from 2 to 10 with an average value of about 6. Polarization sensitivity ratios measured during and just after an adapting flash of polarized light do not differ from that measured in the dark adapted cell. Adaptation with a constant polarized light did not change the ratio although it differentially affected, depending on its orientation, the response amplitude in the two classes of cells. It is concluded that intercellular interaction is not responsible for the apparent enhancement of polarization sensitivity.

Polarization sensitivities were then measured by averaging retinular cell membrane potentials during 5 second flashes of 640 nm monochromatic light. An *effective* flux of between 10 and 100 photons/cell/second was calculated. Averages were compared to responses of the cell to short flashes of higher intensity, and the results indicate that the polarization sensitivity ratio remains constant, even at very low light intensities and leads to the conclusion that the polarization sensitivity ratio and the dichroic ratio are probably he same for the intact cell.

Factors controlling the appearance of spontaneous dark mutants of luminous bacteria. KENNETH NEALSON.

It is common knowledge to those working in the field of bacterial bioluminescence that freshly isolated luminescent forms are often hard to keep in culture as luminous forms. In fact, a great deal of effort is often required to keep the cultures from reverting to dark forms. It is also well known that once a strain of luminous bacteria has been maintained in the laboratory for a long time, this particular problem disappears, and the luminescent form becomes somehow stabilized. We examined this problem by using two well established laboratory strains (designated here as MAV and PF) and eight strains of luminous bacteria freshly isolated from the Woods Hole area. We found that, as reported by Keynan and Hastings, if we grew the newly isolated cultures to stationary phase and allowed them to sit without shaking, that dark forms arose quite easily. With the eight freshly isolated strains, dark colonies appeared usually within twenty four hours, and in all cases, within three days. In all cases, the dark cells became the predominant form in the culture. For MAV and PF on the other hand, darks were never seen until at least two weeks, and then in low numbers. Using a strain of freshly isolated bacteria, we tested many variables to try to gain some insight into the factors which control the conversion of brights to darks. We varied temperature, composition of the medium, and the degree of aeration. The only condition under which the conversion never occurred was under conditions of vigorous aeration. In all cases, the freshly isolated forms converted to dark forms quite easily, suggesting that this is a control mechanism in the natural environment, and possibly a significant one in terms of the ecology of the luminous bacteria.

Protein synthetic rates in pyloric caecae of temperature acclimated starfish, Asterias forbesi. JENNIFER NIELSEN.

A variety of poikilothermic organisms are known to show metabolic compensation in response to temperature changes, which serves to offset direct temperature effects on reaction rates. In toadfish, acclimation to cold is accompanied by an elevation of protein synthesis in liver achieved through more rapid polypeptide chain assembly. Possible compensation in the protein synthetic system with temperature acclimation has now been examined in an echinoderm, *Asterias forbesi.*

Groups of starfish, 19 ± 3 g, were kept in running seawater aquaria at 21° or at 9–11° and were fed mussels for two weeks prior to use. A saline solution of ^{14}C-leucine, 0.025 μCi/arm, was injected into the pyloric duct leading from the stomach to the paired caecae immediately after removal of each arm from the central disc. After incubation of the arms for 10 min in well oxygenated seawater, the caecae were removed and homogenized. ^{14}C-leucine incorporation into protein was determined on filter paper discs, free radioactivity was measured in Aquasol, and total leucine pool was determined by paper chromatography for each of the five arms. Protein synthetic rate was then calculated by use of the specific activity of the leucine precursor pool *in situ.* A study of the time dependence of incorporation indicated protein synthesis in this system is linear with time up to 30 min. Leucine incorporation rate (nmoles leucine/g tissue/min), based on 5 individuals in each group, 5 independent measurements for each, was as follows: 21°-acclimated, 27 ± 13; 10°-acclimated, 59 ± 31. Leucine concentrations in the pyloric caecae (μmoles/g tissue) were 11.8 ± 7.0 and 28.6 ± 8.9, respectively. Net protein synthetic rate in 21°-acclimated starfish, about 1 mg/g tissue/hr, was comparable to that previously found in liver of 20°-acclimated toadfish, measured at the same temperature.

Supported by National Science Foundation Grant No. GB-14570 to Dr. A. E. V. Haschemeyer.

A soluble cell surface material required for spermatozoan-epithelial cell interaction during fertilization in Campanularia flexuosa. MICHAEL G. O'RAND.

Fertilization in *C. flexuosa* is internal. During the fertilization process a capacitation-like interaction occurs between the spermatozoa and the epithelial cells that lead to and surround the eggs. Trypsinization (0.75%, 1 hr, 15° C, pH 7.8) of eggs surrounded by epithelial cells (egg packets) results in loss of fertilizability. Ultrastructural examination of the trypsin-treated egg packets reveals no apparent alteration in fine structure except for the loss of surface coat material from the epithelial cells surrounding the eggs. Epithelial cells of normal (untreated) egg packets have a fuzzy surface coat 200 Å thick. This surface coat is reduced to approximately 50 Å following trypsin treatment. To compensate for the possibility of fewer available epithelial cell surface interaction sites, increasing amounts of sperm were given to eggs in trypsin-treated packets. The fertilization rate increases, but plateaus at 46% implying a saturation of available sites. Trypsinization appears to remove enough of the epithelial cell surface material to prevent complete recovery of the fertilization rate with large numbers of spermatozoa. Epithelial cell surface material may be solubilized by incubation of egg packets in calcium and magnesium free sea water at 4° C for 4 hr on a gyrotory shaker. Centrifugation at 2500 rpm for 30 min results in a supernatant which upon addition of calcium to the normal sea water level, increases the fertilization rate of eggs in trypsinized egg packets. Thus, the necessary spermatozoan-epithelial cell interaction may be described as an interaction between the spermatozoon and a component(s) of the female epithelial cell surface.

Supported by NIH grant HD04543 to Dr. R. L. Miller.

Promiscuous transfer of genetic information from nonmarine to marine bacteria. Tom Patt, Kenneth Nealson and Carolyn Eberhard.

Episomes can mediate rapid transfer of genetic information through a bacterial population. Such transfer has been reported to occur between different strains of enteric bacteria. We have observed that the determinant for utilization of salicylate as carbon source, carried on an episome in *Pseudomonas* sp. (nonmarine), can be transferred into strain MAV of *Photobacterium fischeri* (marine). Likewise determinants for resistance to antibiotics, carried on two different R factors in *Escherichia coli*, were transferred into four luminous and one nonluminous strain of marine bacteria. Intrageneric transfer of an *E. coli* sex factor (F*thy*$^+$) in sea water and enriched sea water media indicates that transfer of genetic information via episomes may occur in the marine environment. Information from dying nonmarine bacteria might thus enter a reservoir in the marine bacterial population.

Effects of changing extracellular ions on photoreceptor potentials in an isolated, perfused vertebrate retina. L. H. Pinto and J. E. Brown.

Intracellular recordings were made from single photoreceptors of the isolated retina of *Bufo marinus* while the ionic composition of the extracellular medium was changed. The light-induced responses of these cells consisted of hyperpolarizations, and were independent of the activity of horizontal cells. Replacement of Na$^+$ by choline$^+$ resulted in membrane hyperpolarization and loss of response to light. Substitution of $CH_3SO_4^-$ for Cl$^-$ or removal of Mg^{++} resulted in no consistent, detectable effect. Removal of external K$^+$ resulted in hyperpolarization and increase in response magnitude, but responses developed an after-depolarization. These results are consistent with the idea that the receptor potential is produced by a decrease in sodium conductance. Lower than normal $[Ca^{++}]_0$ produced membrane depolarization, but during illumination the membrane hyperpolarized to nearly its normal value. Elevated $[Ca^{++}]_0$ produced hyperpolarization; the increment in hyperpolarization produced by light in elevated $[Ca^{++}]_0$ equalled that expected from a steady decrease in sodium conductance by the amount needed to produce the calcium-induced hyperpolarization.

We wish to thank Dr. Arthur Finn for supplying our first toad. This work was supported by the Grass Foundation and NIH Grants EY-00834, EY-00835, EY-00897, and HSAA RR 06067.

Effect of sea star coelomocyte extract on mammalian immuno-competent cells. Robert A. Prendergast.

The coelomocyte of the sea star has previously been shown to contain a protein with remarkable ability to effect cells of the immune response of vertebrates. Delayed inflammatory skin reactions, inhibition of macrophage migration, and activation of macrophages with destruction of a target cell mono-layer are caused by a protein of 32,000 molecular weight. These responses are similar to those of immunized vertebrate thymus-dependent lymphocytes (T-cells) after contact with a specific antigen. The direct role of coelomocyte extract on vertebrate lymphocytes was assessed during short-term tissue culture of human peripheral blood cells with phyto-hemagglutinin, concanavalin A or mixed lymphocyte reactions in the presence of varying concentrations of sea star coelomocyte extract. Tritiated thymidine was added to the cultures 24 hours before termination and the amount incorporated assessed by trichloroacetic acid precipitation of cell contents. Addition of as little as 10 μg of sea star factor (SSF) per ml of medium inhibited PHA stimulation to 50 per cent of control levels. Mixed lymphocyte reaction and concanavalin A stimulation were inhibited by concentrations of less than 1 μg per ml. SSF was not mitogenic in concentrations from 0.01 to 1000 μg per ml. Trypan blue exclusion and ^{51}Cr uptake at the end of the culture period demonstrated equal viability of control and SSF-exposed lymphocytes.

These results indicate that stimulation of T-cell mitosis by alloantigen or lectins can be inhibited by SSF.

This investigation was supported by PHS Training Grant No. GM00265–14 from the National Institute of General Medical Science.

A spectroscope image intensification system for recording in vivo *spectra of bioluminescent organisms.* GEO. T. REYNOLDS, P. BOTOS, JR. AND R. BARBA.

The light emitted by many bioluminescent organisms is so weak that a complete spectrum of a single flash can not be recorded by conventional means. For this reason a prism spectroscope has been designed for use with an image intensifier, so that spectra can be intensified and recorded on film, suitable for conventional analysis. The input lens is fast (f/1.4) in order to collect as much light as possible into the spectroscope. A long focal length output lens spreads the spectral region 3000° to 7000° over the image intensifier cathode. The method is similar to that used by astronomers to observe the spectra of very faint stars.

The system is calibrated for overall spectral response by means of black body radiation at a known temperature. The wave length scale is provided by superposing lines from a high pressure mercury lamp. By means of masks over the input slit the mercury lines can be placed above and below the portion of the field occupied by the spectrum of interest.

Microscopic organisms form their own slit. Macroscopic specimens are coupled to the slit by means of a fiber optics light pipe. By these means a number of *in vivo* spectra have been obtained, agreeing well with observations made by other methods where these exist. Spectra have been obtained for *Pelagia, Clytia, Parazoanthus, Obelia, Acanthoptilum, Renilla, Ptilosarcus, Mnemiopsis* (adult and embryo), *Chaetopterus* (adult and embryo), *Harmothöe, Porichthys,* and the fireflies *Photinus pyralis* and *Photuris pennsylvanicus.* The *in vivo* spectrum of *Porichthys* exhibited a bimodal distribution, whereas the *in vitro* spectrum did not.

This research was supported by AEC Contract AT (30–1)–3406.

A near ultraviolet photoproduct of tryptophan inhibits the mitosis and development of Arbacia *eggs.* RONALD C. RUSTAD, BLENDA C. ANTONELLIS AND SEYMOUR ZIGMAN.

Irradiation of tryptophan with near ultraviolet light is known to produce a series of photoproducts resulting from the splitting and recondensation of the pyrrole ring. Two tryptophan photoproducts with molecular weights of 12,000 (PT1) and 425 (PT2) were isolated by filtration with Sephadex G10. The low molecular weight substance (PT2) was employed in the present investigation because the higher molecular weight compound would not be expected to penetrate the fertilization membrane of the sea urchin egg. PT2 has been shown to contain a free radical.

Incubation of fertilized eggs in PT2 resulted in a concentration dependent delay of the first two cell divisions. The first cell division was more delayed than the second. The delay of the first division was not materially different if the exposure to PT2 was initiated ten minutes before fertilization or five minutes after fertilization. In some experiments a concentration of 1 μg/ml blocked the first cell division. Continued exposure of the embryos to the photoproduct resulted in delays or complete blocks of development in the blastula, gastrula or early pluteus stages. However, when the treated embryos were washed at the four-cell stage there were no obvious effects on late development.

This research was supported by Contract W-31–109-ENG-78 with the U. S. Atomic Energy Commission and the National Eye Institute (EY 00459).

Thermodynamics of living Chaetopterus *oocyte spindles–polarizing microscope measurements with a new hydrostatic pressure chamber.* EDWARD D. SALMON.

Changes in spindle birefringence (BR), morphology, and chromosome position in the meiotic metaphase arrested spindle of live *Chaetopterus* oocytes were observed directly under hydrostatic pressures up to 6000 psi. For this, a small controlled-temperature pressure chamber was developed which uses strain-free optical glass for the windows, adapts directly to a standard compound microscope, and sustains working pressures up to 12,000 psi. At a given pressure, spindle BR reversibly fades to an equilibrium value; with a short delay, spindle length also decreases to an equilibrium size with one pole remaining attached to the cell surface. Under phase contrast the chromosomes are seen to move towards the attached pole as the spindle shortens. Release of pressure results in full spindle recovery. The rate of BR decay increases with increased pressures. The rates of spindle shortening and chromosome movement also increase, but only up to a certain pressure. At 4000 psi BR disappears within 1 minute while the chromosomes move at about 15 microns/minute all the way to the cell surface. At 6000 psi BR disappears within 15 seconds. However, there is no visible shortening of the spindle and the chromosomes do not move. The equilibrium pressure BR relationship is consistent with Inoué's model of *in vivo* spindle microtubule assembly based on an equilibrium temperature BR relationship which involves first order polymerization-depolymerization kinetics. Thermodynamic analysis of pressure data at both 22° and 17.5° C yielded a partial molar volume change of 400 ± 25 ml/mole for the BR polymerizing unit. This value is comparable to those found for actin, TMV, and other proteins polymerized by entropically driven reactions *in vitro*. The pressure data, with previous temperature data, strongly suggest that the mechanism of spindle microtubule assembly *in vivo* is dependent upon hydrophobic bonding and release of bound water.

Supported in part by grant CA10171 and training grant GM00606 from NIH, and grant GB31739X from NSF.

A large change in axon fluorescence, potentially useful in the study of simple nervous systems. B. M. Salzberg, H. V. Davila, L. B. Cohen and A. S. Waggoner.

We have found a rather large, fast change in extrinsic axon fluorescence which could provide a powerful technique for measuring membrane potential in systems where, for reasons of scale, topology, or complexity, the use of electrodes is inconvenient or impossible. More than 150 fluorescent dyes were studied, many of which, when applied to the axolemma, exhibited changes in absorption or emission during activity. Most of these changes were small, and the detection required signal averaging. Gary Strichartz suggested that we explore the class of merocyanine dyes which are known to have extraordinary solvent indicator properties. One merocyanine, 5-[1-N-propylsulfonic acid-benzoxazole-2-tetramethin]-1,3-dibutyl-2-thiobarbituric acid, provided by the Eastman Research Laboratory, gives a rather large signal, easily visible in a single oscilloscope sweep. A cleaned giant axon from the squid, *Loligo pealei,* was mounted in a chamber and bathed for ten minutes in a 25 μg/ml dye-seawater mixture. The mixture was then replaced with nitrogenated seawater and the stained axon was illuminated at 540 ± 25 nanometers. The fluorescence was measured at right angles through a 590 nm barrier filter. During a spike, the intensity increase represented a change in the resting fluorescence of one part in 10^3 and the signal to noise ratio was about 10:1 in a single sweep. Optical measurements during voltage clamp demonstrated that the change in axon fluorescence depended linearly upon membrane potential rather than upon the ionic currents or the conductance changes that occur during the action potential. We hope to use this fluorescence signal to study integrative behavior in invertebrate ganglia by constructing a computer linked array of photodetectors capable of monitoring activity in, perhaps, 100 cells simultaneously.

Supported by Public Health Service grant numbers NS 08437 and NS 10489.

Toxic effects of fuel oil on haustoriid amphipods and pagurid crabs. D. M. Sandberg, A. D. Michael, B. Brown and R. Beebe-Center.

An intertidal benthic amphipod, *Neohaustorius biarticulatus* and the hermit crab *Pagurus longicarpus* were exposed to various concentrations of #2 fuel oil under laboratory conditions. One hundred milligrams of oil was introduced to 1 liter of synthetic sea water and the mixture stirred (magnetic stirring) twice daily for 30 minutes. LD 50's in solutions stirred at differing rates were compared. Five hundred milliliters of solutions of oil in sea water at concentrations of 50, 25 and 12.5 mg/l were placed in 1 liter flasks. Six individuals of *N. biarticulatus* were introduced to each flask. Toxicity due to turbulent stirring (25–33% vortex) was significantly greater than with slow stirring (no vortex). LD 50's occurred in a majority of experiments in less than 36 hours at 50 and 25 mg/l in turbulently stirred oil compared to 72 hours for slow mixing conditions. In a separate experiment, clean sand was stirred with the oil-sea water mixture. Twenty milliliters of oiled-sand was used in each test flask with the oil-sea water mixture while control flasks had clean sand and synthetic sea water. Amphipods initially burrowed normally into the oiled-sand but in less than 12 hours the animals no longer burrowed (*i.e.,* remained on the sand surface) at 50 mg/l and all were dead within 24 hours. Specimens of *Pagurus longicarpus* were kept in 200 ml solutions in finger bowls (4 individuals per 300 ml bowl) and exposed to the same oil/sea-water concentrations as above. LD 50's occurred within 12 hours at 50 mg/l. A pre-lethal effect was consistent throughout. The animals vacated their shells and appeared incapable of coordinated movement several hours before death. Since oil is affected by evaporation, dissolution and chemical or biological attack with time after an oil spill, the effects of length of time of stirring the oil in the laboratory on toxicity levels was tested. Toxicity decreases with increasing age of oil/sea-water mixture (1–7 days old).

Development of single cells from mechanically-disrupted thalli of Prasiola stipitata *Suhr.* JEROME A. SCHIFF, RALPH S. QUATRANO, GARY C. HARRIS, MARNIE LEGG AND JONATHAN STALEY.

Hand homogenization of thalli of *Prasiola stipitata* Suhr releases single cells (SC) almost quantitatively leaving behind a honeycomb of empty cell walls. The SC still retain what appears to be the innermost wall of the thallus since they do not burst in hypotonic media and display a distinct outer layer which separates from the protoplast on plasmolysis. The SC rapidly attach to glass, plastic or other surfaces by secreting a mucilagenous material which displays metachromatic staining with toluidine blue under acidic conditions indicating the presence of a highly acidic—possibly sulfated—polysaccharide. The attached SC can be treated with an antibiotic mixture to remove contaminating materials and organisms. The axenic attached SC develop into filaments on Lewin's defined medium by successive divisions and, subsequently, two-dimensional growth occurs by the formation of transverse walls. Differentiation of blade and rhizoid eventually occurs. The pigment content and composition are typical of green algae and are essentially the same in thalli and SC. The photosynthetic rate does not change appreciably on producing SC from thalli, and treatment with antibiotics does not significantly affect pigmentation, photosynthesis, or subsequent growth and differentiation of the organism as far as they have been observed (about 7 weeks from attachment). The growth of SC appears to offer an opportunity to clone single cells from the diploid stage of *Prasiola,* on a defined medium axenically, and will be useful in studies of polarity, polysaccharide biosynthesis and chloroplast development. If the haploid stage (which gives rise to gametes) can be similarly cloned, the low chromosome number of the organisms suggests that genetically-mapped pure mutant strains may be obtainable.

We are indebted to Dr. J. Hellebust for suggesting the use of *Prasiola,* and to Dr. M. Wynne for helping us to locate and identify it.

A trypsin-like enzyme in cortical granules of sea urchin eggs, and its role in fertilization. H. SCHUEL, L. LORAND, K. CHEN AND W. L. WILSON.

Cortical granules were isolated from homogenates of unfertilized *Strongylocentrotus purpuratus* eggs by zonal centrifugation. Acid aryl esterase activity, substrate: β-naphthyl acetate, showed a bimodal distribution in the sucrose density gradient, with one activity peak in the cortical granule fractions and the other in the microsomal fractions. The cortical granule esterase proved to be a trypsin-like enzyme, since it also hydrolyzed α-N-benzoyl-L-arginine ethyl ester (BAEE), and was completely inhibited by soybean trypsin inhibitor (SBTI). This enzyme represented 50% of the total aryl esterase activity in the egg. The microsomal esterase was not inhibited by SBTI. By these criteria the cortical granules contain 100% of the trypsin-like activity.

The elevation of the fertilization membrane of *Arbacia punctulata* eggs was inhibited when they were fertilized in the presence of trypsin inhibitors SBTI (0.25 mg/ml) or tosyl lysine chloromethyl ketone (TLCK) at $1\text{–}50 \times 10^{-4}$ M. These eggs were also polyspermic. Normal monospermic fertilization was obtained when the eggs were inseminated in sea water following a 5 min exposure to TLCK. These observations indicate that the trypsin-like enzyme in sea urchin egg cortical granules is involved in the elevation of the fertilization membrane and the establishment of the block to polyspermy at fertilization. Other observations suggest that the enzyme may also participate in the activation of protein synthesis within the egg (Runnström, 1966, *Advan. Morphogen.*, **5**: 221; Grossman and Troll, 1970, *Biochim. Biophys. Acta*, 212: 192).

Supported by grants NIH 5K4-CA-8608 and Amer. Cancer Soc. P-616 and NIH contract 69-2202 to H. S., and by NIH grants 5K6-HE-3512, HL-02212, GM-17939, and 5T1-GM-626 to L. L.

A role for the follicle cells during vitellogenesis in the squid Loligo pealei. KELLY SELMAN AND ROBIN A. WALLACE.

The study of yolk in cephalopods has been limited to Fujii's (1960) analysis of cuttle-fish eggs in which he reported a large ($M = 2.5 \times 10^6$) lipoprotein present in water extracts after centrifugation at 105,000 × *g* for 3 hours. We have also found a major, soluble high-molecular weight component in similar preparations from oviducal *Loligo* eggs (evaluated in the ultracentrifuge on 4% polyacrylamide gels). However, the material comprises only 20% of the total egg protein; the remaining protein was present in the insoluble pellet obtained by centrifugation of the initial extract and could only be solubilized with 2% SDS and 5 mM dithiothreitol. The insoluble fraction as well as the soluble material resolved into at least four apparently identical bands when run on 4% polyacrylamide gels in the presence of SDS. Thus, the insoluble fraction may represent a more highly cross-linked form of the soluble protein.

Growing oocyte-follicles (0.9 mm diameter) were incubated in blood containing antibiotics and ^{3}H-leucine. Label was incorporated into protein with essentially linear kinetics for six hours, after which the oocyte-follicles were washed and the insoluble fraction (containing 92% of the total protein) was analyzed on SDS-polyacrylamide gels. Three of the bands on the gel were specifically labeled, indicating endogenous synthesis of these peptides. Oviducal eggs showed no incorporation of label into any component when treated similarly.

Light microscope observations implicated the follicle cells as the site of synthetic activity. These cells initially proliferate to form finger-like folds around the immature oocyte and display cytoplasmic basophilia prior to vitellogenesis. As yolk subsequently accumulates within the oocyte, the folds of follicle cells become distended around the periphery of the oocyte. Autoradiograms were made of the isolated oocyte-follicle incubated with ^{3}H-leucine for various lengths of time. These showed that the follicle cells were the major site of protein synthesis and that material initially synthesized in the follicle cells appeared to be transferred subsequently into peripheral yolk granules within the oocyte.

This study was performed in the Fertilization and Gamete Physiology Training Program at the MBL (grant NIH 5-T01-HD00026-11).

Effects of varied oxygen tension on properties of excised squid giant axon. D. R. SHANKLIN, S. A. STEIN, D. THOMPSON AND J. BANKS.

Excised giant axons remain stable and capable of conducting action potentials for varied periods, their decay often rightly attributed to injury during removal. With both stimulating and recording external electrodes in air and the bulk of the nerve in seawater the thresholds for voltage and duration eventually show a progressive rise. These rises have been examined in the light of change in oxygen tension mostly with gas flowing at 3–4 l/min. Most decay curves of duration threshold in μS/min at constant 1.5V stimulus show biphasic form. The first phase is directly related to the oxygen content according to the formula $m_1 = 0.017P + 0.62$ where P is the effective percentage of oxygen in the gas bathing the electrodes. The second phase has an accelerated slope which relates to the slope of phase one by $\log_{m_2} = 0.5000\ m_1 + 0.4000$. The decay is thus set by the oxygen tension of the environment in conformity with the "oxystat" concept (Shanklin, 1971, *Int. J. Clin. Pharm.*, **5**: 20–25). Conduction velocity is also affected. Using a relative scale equating all velocities prior to application of flowing gas as 1.0 there is a rather uniform effect at all concentrations during phase one; in phase two the velocity is roughly proportional at 21 or more per cent oxygen, with 90–100% oxygen having the highest velocity. Comparisons with stationary air indicate a clear accelerating effect of gas flow with nerves in stationary air showing a first phase 3–4 times longer. Changes in action potential seem to involve all phases of membrane current with negative action potentials showing early depression.

Supported by a grant from the John A. Hartford Foundation, New York, New York.

The effects of mercury salts on sea urchin gametes. JERRY W. SHAY AND GERTRUDE W. HINSCH.

Effects of mercuric chloride (MC) and methyl mercuric chloride (MMC) on *Arbacia punctulata* gametes were studied. One and one-half hours after insemination 100% of the eggs cleaved in the presence of 0.25 parts per million (ppm) MC whereas only 22% cleaved in 0.25 ppm MMC. MC appeared to enhance membrane elevation while MMC did not. After 1½ hours MC (0.50 ppm) resulted in 100% membrane elevation and 12% cleavage while MMC (0.50 ppm) resulted in 7% membrane elevation and no cleavage. MC (1.00 ppm) produced 88.5% membrane elevation and no cleavage while MMC (1.00 ppm) produced no membrane elevation and no cleavage. The pigment granules in *Arbacia* eggs were clumped and extruded by solutions of MC but not MMC. Fertilized eggs removed from mercury solutions after different periods of time and returned to filtered sea water, cleaved after incubation up to 27 minutes in 1.00 ppm MC but not after 3 minutes in 1.00 ppm MMC.

Since spironolactone and DL-penicillamine have been used in treatment of heavy metal poisoning, solutions containing 20 ppm spironolactone, fertilized eggs and various concentrations of mercury salts were made which exhibited higher per cent cleavage after 1½ hours than those in mercury but without spironolactone. Penicillamine (0.01%) resulted in approximately the same per cent cleavage after 1½ hours as the controls without penicillamine and without mercury. Mercury salts (5.00 ppm, normally inhibitory) and 0.01% penicillamine allowed 84% cleavage in MMC and 100% in MC. Concentrations of mercury salts (5.00 ppm) which inhibit sperm motility immediately and 0.01% penicillamine resulted in durations of motility equivalent to controls.

Ultrastructural studies showed electron-dense granules (0.25 μ) in the midpiece of spermatozoa treated with MMC but not in MC or controls. Likewise a layer of electron-dense material was on the outer membrane of MMC treated eggs but not MC or control eggs. Mercury salts and most other heavy metals may inhibit biological systems by binding to certain proteins.

This study was performed in the Fertilization and Gamete Physiology Training Program at the MBL (NIH grant 5-T01-HD00026–11).

RNA-directed DNA polymerase activity in sea urchin development. CHI-HUNG SIU, MARGARET SEDENSKY AND MARCO CRIPPA.

RNA-directed DNA polymerase activity has now been detected in a number of eucaryotic systems. It has been suggested that this enzyme activity may play an important role in cell differentiation. We have studied the embryos of *Arbacia punctulata* and have devised methods to separate the different DNA polymerases.

Chromatin was isolated from embryos at the 16-cell stage and solubilized by sonication. Rifampicin derivatives, AF/ABDPcis and AF/AP, both showed significant inhibitory effect on the total polymerase activity in this fraction. When sedimented on a glycerol gradient and assayed for endogenous activity, two peaks of DNA polymerase activity were observed. The slower sedimenting peak was sensitive to AF/ABDP and to RNAse treatment. The sensitivity to RNAse treatment strongly suggests that this slow sedimenting DNA polymerase activity was dependent on endogenous RNA. RNA-dependent DNA polymerase activity was also detected in unfertilized eggs and hatched blastulae by the same method.

Fractionation of DNA polymerases was attempted on a DEAE-cellulose column. Three peaks of DNA polymerase activity were obtained. The first in the flow-through of the column, the other two eluted at 0.15 M and 0.3 M $(NH_4)_2SO_4$. The different peaks were assayed for drug sensitivity and stimulation by different templates. The first peak was sensitive to drug and to RNAse treatments, while the other two behaved as normal DNA polymerases. DNA polymerase activity in peak I was also stimulated by poly rA (primed by oligo dT) which, however, was not used as template by peaks II and III.

Therefore, we conclude that there is present in the developing sea urchin embryo RNA-directed DNA polymerase, which can be distinguished from DNA-dependent DNA polymerases by differential chromatin association, drug and RNAse sensitivity, template specificity, and elution pattern from DEAE-cellulose column.

This study was performed in the Fertilization and Gamete Physiology Training Program at the MBL (NIH grant 5-T01-HD00026-11).

Larvae of strigeid trematodes in the Woods Hole area. HORACE W. STUNKARD.

Cercaria nassa Martin, 1945 from *Ilyanassa obsoleta* is the only strigeid cercaria described from marine molluscs of the Atlantic coast of the United States. It closely resembles *Cercaria scudderi* Olivier, 1941 from the pulmonate snail, *Lymnaea palustris elodes,* taken in northern Michigan. The life-cycle of the latter species was elucidated by Hoffman and Hundley (1957, *J. Parasitol.,* **43**: 613–627) who found metacercariae in the brain of the freshwater stickleback, *Eucalia inconstans,* raised adult forms in chicks, incubated the eggs, obtained miracidia, infected laboratory reared snails, and got cercariae with which they infected sticklebacks. Dubois (1966, *Bull. Soc. Neuchâtel. Sci. Natur.,* **89**: 19–56) predicated identity of these cercariae and the cercaria described by Olivier and identified the species as *Diplostomum scudderi* (Olivier). Strigeid metacercariae occur as natural infections on the brain of *Fundulus heteroclitus* in the Woods Hole area. Attempts to infect *F. heteroclitus* by exposure to *C. nassa* were futile. Feeding of metacercariae from the brain of *F. heteroclitus* to laboratory reared birds: snowy egrets, *Egretta thula;* the black crowned night heron, *Nycticorax nycticorax;* cormorants, *Phalacrecorax auritus;* gulls, *Larus argentatus;* and day-old chicks, gave only negative results. These experiments demonstrate that *C. nassa* is not related to the metacercaria on the brain of *F. heteroclitus,* but other stages in the life-cycles of the two species remain quite unknown. *Cercaria nassa* belongs to the larval group, Diplostomulum, whose adult stages occur primarily in birds and belong to the family Diplostomidae. Development of the metacercariae on the brain of *F. heteroclitus* shows a complete metamorphosis, with regression of all cercarial structures and the evolution of new tissues and organs. The formation of a bilobed tribocytic organ and medial cotylae identify the larvae as members of the larval group, Tetracotyle, whose adults belong in the family Strigeidae.

Differential developmental potential of echinoderm eggs in sea water from various sources in and near the Marine Biological Laboratory, Woods Hole. GAIL SUSAN TUCKER, MARGARET C. HILL AND CHARLES R. WYTTENBACH.

Quantitative and qualitative measures were made of the developmental potential of sea urchin (*Arbacia*) and starfish (*Asterias forbesi*) eggs in sea water collected from taps in each of the three MBL laboratory buildings, from the end of the MBL intake dock in Great Harbor, and from Nobska Point. Batches of about 100 eggs per sea water sample (fertilized in a concentration of 10^6 sperm/ml) were compared at one time; all samples were tested at least in triplicate over a span of several weeks. Notations were made of per cent normal fertilization, per cent of fertilized eggs which cleave, and per cent of cleaving eggs which yield normal plutei. Additionally, observations on the rate of fertilization, incidence of polyspermy, and occurrence of asynchronous development were made.

Differences among the water samples were seen consistently, and in virtually all cases the samples displayed the same relative ranking regardless of the criterion used. Samples from the three MBL sources (all pH 8.0) differed considerably in their capacity to support development (*e.g.*, Loeb Building, 76% fertilization; Lillie Building, 51% fertilization). Nobska water, pH 7.8 or 8.2 (natural variation between different collections) was the best (87% fertilization) and dock water, pH 7.57, was the poorest (36% fertilization) among the "natural" samples tested. However, all MBL samples as well as dock water become considerably enhanced in potential by adjusting their pH to 7.80 (*i.e.*, fertilization became about 95%). A series of sperm dilution tests revealed that increasing the sperm concentration to about 10^7/ml enhanced the incidence of fertilized eggs in those water samples having the lowest fertilization percentages; however, this did not produce a corresponding increase in the proportion of normally cleaving eggs.

The results demonstrate that the composition of the sea water is a significant factor in the successful fertilization and development of echinoderm eggs, and therefore in the interpretation of data on their development. pH is a pertinent factor, especially in the MBL flowing sea water system; the data suggest the desirability of perhaps reducing its pH to 7.80. However, as the comparison of different Nobska samples revealed, other factors, as yet unidentified, are also of importance.

Supported by Biomedical Sciences Support Grant RR-07037 to the University of Kansas.

Assembly of microtubules and aster-like structures in homogenates of Spisula solidissima *oocytes.* R. C. WEISENBERG, G. G. BORISY AND J. B. OLMSTED.

Oocytes of the surf clam, *Spisula solidissima,* were artificially activated and at the desired stage were washed twice in 1 M glycerol, pH 8, and then homogenized in one volume of cold 0.5 M 2(N-morpholino)ethane sulfonic acid (MES) or 0.1 M piperazine-N-N′-bis(2-ethane sulfonic acid) (PIPES) at pH 6.5 containing 1 mM EGTA, and in some experiments, 0.5 mM $MgCl_2$ and 1 mM GTP. Microtubule formation was assayed by electron microscopy of aliquots of the homogenates diluted 1:10 with warm buffer solution and negatively stained with 1% uranyl acetate. Formation of aster-like structures was assayed by light microscopic observation of aliquots of homogenates diluted 1:10 with 1 M hexylene glycol solution, pH 6.2. No microtubules or asters were observed as long as the homogenates were maintained at 0° C, but rapidly formed (within 10 min) upon raising the temperature to 35° C. Microtubule formation was slower and less extensive at room temperature. Microtubule formation occurred in homogenates of both activated and unactivated oocytes; however, in homogenates of activated oocytes in which nuclear membrane breakdown had occurred, microtubule polymerization developed in a highly organized pattern. Bundles of microtubules were observed (by both light and electron microscopy) radiating out from a central, dense spot, forming a large structure (up to 50 μ in diameter) which resembled a spindle aster. No aster-like structures appeared in homogenates of unactivated oocytes. Centrifugation of the homogenates at 25,000 × g for 10 min produced supernatants which were not competent to form either microtubules or aster-like structures. The pelleted material from homogenates of metaphase oocytes induced aster formation when mixed with either activated or unactivated oocyte supernatants; however, pellets prepared from unactivated oocytes did not induce aster formation. It appears, therefore, that a microtubule organizing center (MTOC) is formed or becomes functional in *Spisula* oocytes shortly after activation, while the subunits are present and capable of polymerization into microtubules at all stages.

Supported by grants GB-24706 (R. C. W.) and GB-8735 (G. G. B.) from the National Science Foundation.

Metchnikoff revisited: introducing the dogfish to gout and tissue injury. GERALD WEISSMANN AND ROY J. SOBERMAN.

Release of lysosomal enzymes (cytases of Metchnikoff) from phagocyte granules results in tissue injury and inflammation. These events can be conveniently studied in *living* buffy coat phagocytes of the dogfish (*Mustelus canis*) since (1) the granules' size (0.7–0.8 μ) permits light microscopy, (2) the granules stain supravitally with toluidine blue, and (3) they constitute a uniquely homogeneous population. As a model for gout in the human, cells were exposed to crystalline monosodium urate (MSU, 20 mg/ml). For the first half hour, crystals were taken up in phagocytic vacuoles, the contents of which stained *ortho*chromatically after fusion with *meta*chromatic primary lysosomes. After 30–60 minutes, these secondary lysosomes degranulated, releasing sudden, explosive waves of dye into the cytoplasm. Thereafter, crystals remained in agonal cells which were devoid of dye, and finally became extruded. Cells released cytoplasmic lactate dehydrogenase 15 min before beta-glucuronidase (from lysosomes), a sequence consistent with the cinemicroscopic findings (obtained through the courtesy of John

M. Arnold), and in contrast to preferential release of beta-glucuronidase after ingestion of the inert particle, zymosan. Since phagocytes also discharge lysosomal enzymes without undergoing cell death upon encounter with immunoglobulins (Ig's) on surfaces, eggs of *Arbacia punctulata* were coated by heat-aggregated shark plasma Ig's and exposed to dogfish phagocytes in complement-depleted plasma. Within 15 min, over 60 per cent of eggs underwent partial or complete lysis (*vs* 4 per cent of controls) after first raising classical fertilization membranes. Appropriate cell movement and degranulation of the phagocyte was required: both colchicine (6 μM) and cytochalasin B (2 μg/ml) inhibited phagocyte-induced cytolysis. Adequacy of Ig coating was determined by lysis of all eggs with complement in the absence of phagocytes. These experiments support the validity of the "suicide sac" hypothesis of gouty inflammation, and the "reverse endocytosis" mechanism for phagocyte-mediated immune tissue injury.

How near UV photoproducts of tryptophan inhibit dogfish Mustelus canis *lens protein synthesis.* SEYMOUR ZIGMAN AND TERESA YULO.

Previously, near UV irradiation of tryptophan (trp) in neutral solutions was found to produce photoproducts resulting from pyrrole ring splitting and recondensation. Two photoproducts have now been characterized, and their effects on *in vitro* uptake of 14C amino acids (AA) into acid soluble and protein phases of dogfish lenses is herein reported. *In vitro* incorporation of 14C -AA's into lens crystallins was formerly reported to be markedly inhibited by trp in the presence of near UV light.

Fresh lenses were incubated for 24 hr at 22° C in elasmobranch Ringer's medium with and without purified trp photoproducts, and in the presence and absence of 3000 μW/cm^2 of near UV light (80% at 365 nm). 14C -AA mixture (2.5 μCi/10 ml medium) was then added, and incubation continued for another 24 hr. Trp photoproducts with molecular weights of 12,000 (*PT1*) and 425 (*PT2*) isolated by G10 sephadex filtration were used at 100 μg/ml. After incubation, lens capsules were removed, and cortices were disrupted with a magnetic stirrer. Radioactivities of the TCA-soluble pool and the TCA insoluble proteins were estimated. Capsules soaked 2 days in distilled water were then dissolved in 1% NaOH. Fluorescence at 440 nm (360 nm excitation) and radioactivity were measured. Crystallins were purified with DEAE-cellulose.

Both PT1 and PT2 were bound to lens capsules. UV exposure increased their brown color and fluorescence. Only 14C-PT2 penetrated the lens capsule to cause yellowing and to impart radioactivity to the cortical soluble pool and proteins. Near UV exposure inhibited the uptake of 14C -AA's by cortical acid soluble and protein phases. PT1 and PT2 in the dark inhibited incorporation to the same extent as UV light alone (50%), but lenses incubated with PT1 or PT2 in UV light incorporated only 25% of the dark controls. Capsules of lenses UV irradiated with PT1 and PT2 retained twice the radioactivity of the dark controls, indicating strong amino acid binding. The incorporation of AA's into all crystallins was decreased during incubation with PT2 in near UV light. Alpha AA incorporation was inhibited by as much as 97%.

It appears that trp photoproducts (especially in near UV light) bind to lens capsules thus blocking AA entry into the cortex and reducing the supply of AA's for protein synthesis.

Supported by National Eye Institute (EY 00459) and Rochester Eye Bank.

Vol. 143, No. 3 December, 1972

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

OVARIAN DEVELOPMENT IN *HABROBRACON JUGLANDIS* (ASHMEAD) (HYMENOPTERA: BRACONIDAE). I. THE ORIGIN AND DIFFERENTIATION OF THE OOCYTE-NURSE CELL COMPLEX

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Many hymenopterans are characterized by arrhenotoky, a type of sex determination where unfertilized eggs give rise to haploid males. Since all recessive mutations should express themselves in the impaternate male, all the chromosomes donated by a mutagenized female can be scored for mutations in the hapoid males that receive them. In such studies of mutagenesis the parasitoid braconid wasp, *Habrobracon juglandis,* has long been a favorite laboratory animal, and a considerable body of genetic information has accumulated during the 50 years it has been studied (see review by Whiting, 1961).

In *Drosophila melanogaster,* the insect species for which the most genetic information is available, oogenesis has been extensively investigated at both the light and electron microscope levels, and many insights into the developmental genetics of the ovary have resulted from studies of the aberrant forms of oogenesis characteristically seen in females possessing certain mutant genes that markedly influence their fertility (see review by King, 1970).

Before similar investigations can be attempted on the developmental genetics of the *Habrobracon* ovary, the details of normal ovarian development must be worked out. This paper reports the results of combined light and electron microscope studies of several aspects of normal oogenesis in young adult wasps.

MATERIALS AND METHODS

The ovarian material was obtained from newly eclosed female wasps from the Whiting wild type stock 33 reared at 30°C upon larvae of the Mediterranean flour moth, *Ephestia* (=*Anagasta*) *kuhniella.* Feulgen-stained whole mounts were made of ovaries using the procedure of King, Burnett and Staley (1957, page 242). To produce plastic embedments, ovaries were fixed (first with glutaraldehyde and then osmium), dehydrated, and embedded in Maraglas according to previously described methods (Cassidy and King, 1969). Sections were cut on

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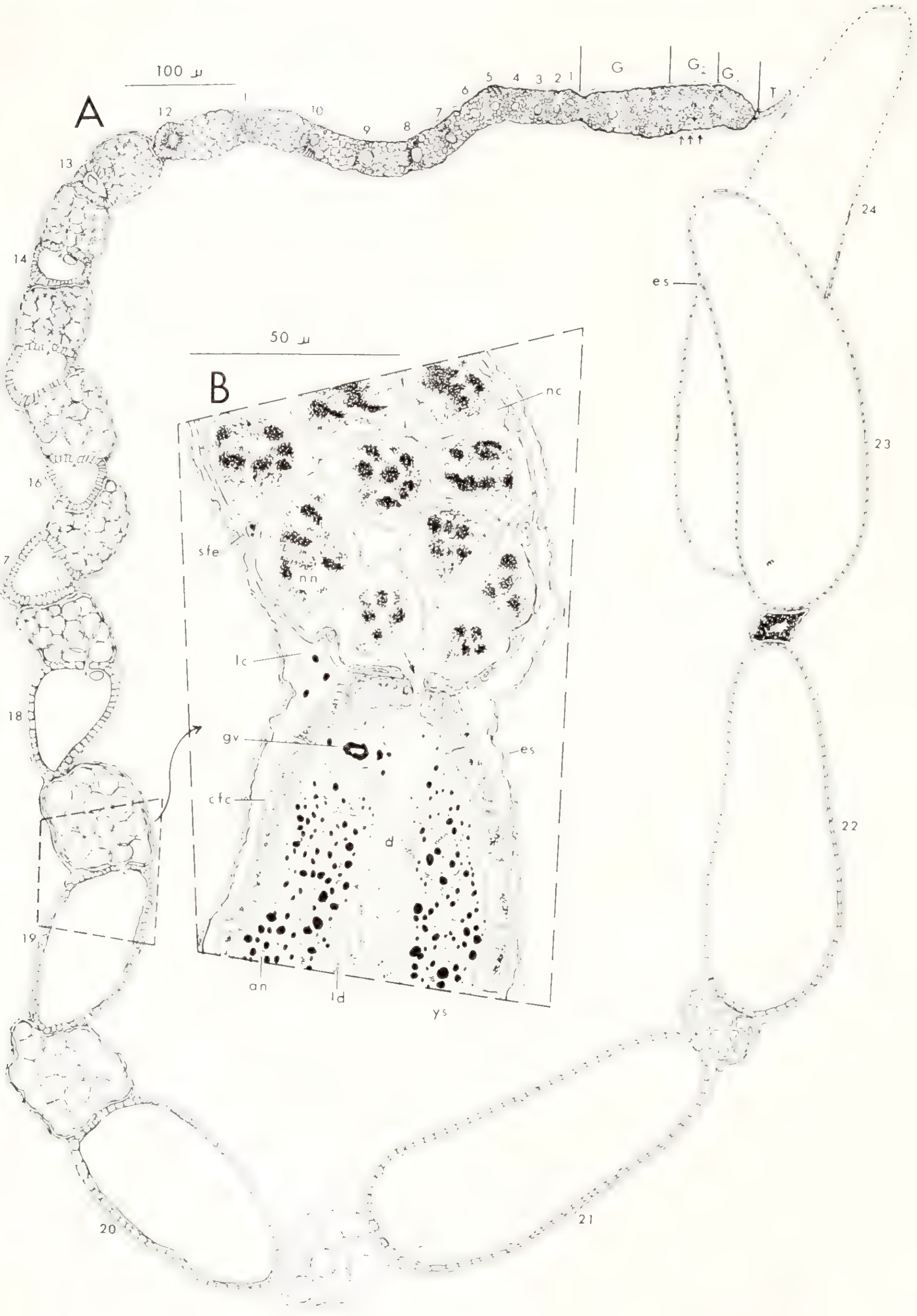
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FIGURE 1.

glass knives using an LKB Ultrotome. A few 1 μ thick sections were always cut before and following a series of thin sections. Some of these thick sections were used in cytochemical studies employing the periodic acid-Schiff (PA S) procedure for polysaccharides, fast green (at pH 2) for proteins, and azure B (at pH 4) for nucleic acids (see King, 1960 and King and Koch, 1963, for the rationale and technical details of the procedures used).

Sections giving gray interference patterns were picked up in groups of 8–10 upon Formvar-carbon coated, one hole, copper grids using the LKB section collector-stereoscope assembly. The sections were stained with uranium and lead as described previously (Cassidy and King, 1969). Approximately 1000 electron micrographs were taken using a variety of electron microscopes (a Siemens Elmiskop Ia, operated at 60 kV; an Hitachi HU11A, operated at 50 kV; or an AE1 801, operated at 60 or 80 kV).

The drawings of egg chambers at various developmental stages presented in Figures 1A and B were made from whole mounts or sections using a Wild M20 light microscope equipped with a drawing tube. In situations where information concerning the three-dimensional morphology of submicroscopic organelles was sought, tracings were drawn from composite electron micrographs made with overlapping prints of specific areas in serial, ultra-thin sections. The tracings were then positioned above one another and separated by glass spacers of appropriate thickness. By viewing the stacked tracings simultaneously above a light box, it was possible to visualize various organelles in three dimensions.

OBSERVATIONS AND CONCLUSIONS

The light microscopic morphology of the ovary

The reproductive system of the female *Habrobracon* contains four ovarioles, each of which is divided into a distal germarium and a proximal vitellarium (Fig. 1A). A terminal filament extends from the anterior edge of the germarium and joins the terminal filament of the other ovariole (see Genieys, 1925, Fig. 2).

The *Habrobracon* germarium contains an anterior region (Fig. 1A, G_1 which

FIGURE 1 (A). A drawing of a single ovariole of *Habrobracon juglandis*. The germarium is divided into three regions (G_1–G_3, see text) and is connected to a terminal filament (T). Arrows point to dividing cystocytes in the mid-germarial region. The vitellarium contains a linear array of 24 oocytes. The boundaries of the follicle cells are outlined and so are the nuclei of some of the nurse cells. In seven egg chambers arrows mark the position of canals through which cytoplasm is transferred from the nurse cells to the oocyte. While such canals exist in earlier chambers, they are closed off in more posterior ones. The nurse cells associated with oocytes 22 and 23 have degenerated, forming a cap of debris. The nuclear envelope is breaking down in oocyte 22, and oocytes 23 and 24 are in the first meiotic metaphase. The ovariole is surrounded by an epithelial sheath (es), and its dilated, posterior portion serves as a storage chamber for the mature eggs (oocytes 23 and 24). (B). A drawing of a portion of egg chamber 19 at higher magnification. Nurse cells are interconnected and are characterized by multiple nucleoli. Cytoplasm passes into the oocyte through a canal (arrow) connecting it with adjacent nurse cells. The germinal vesicle contains a conspicuous nucleolus and is intermediate in size between the nurse and follicle cell nuclei. The ooplasm contains accessory nuclei (an), lipid droplets (ld), and yolk spheres (ys). A monolayered squamous follicular epithelium (sfe) encloses the nurse subchamber, while the oocyte is enclosed by a monolayer of cuboidal follicle cells (cfc). Abbreviations are: es, epithelial sheath; d, delta; lc, lumen cell. See text for further discussion.

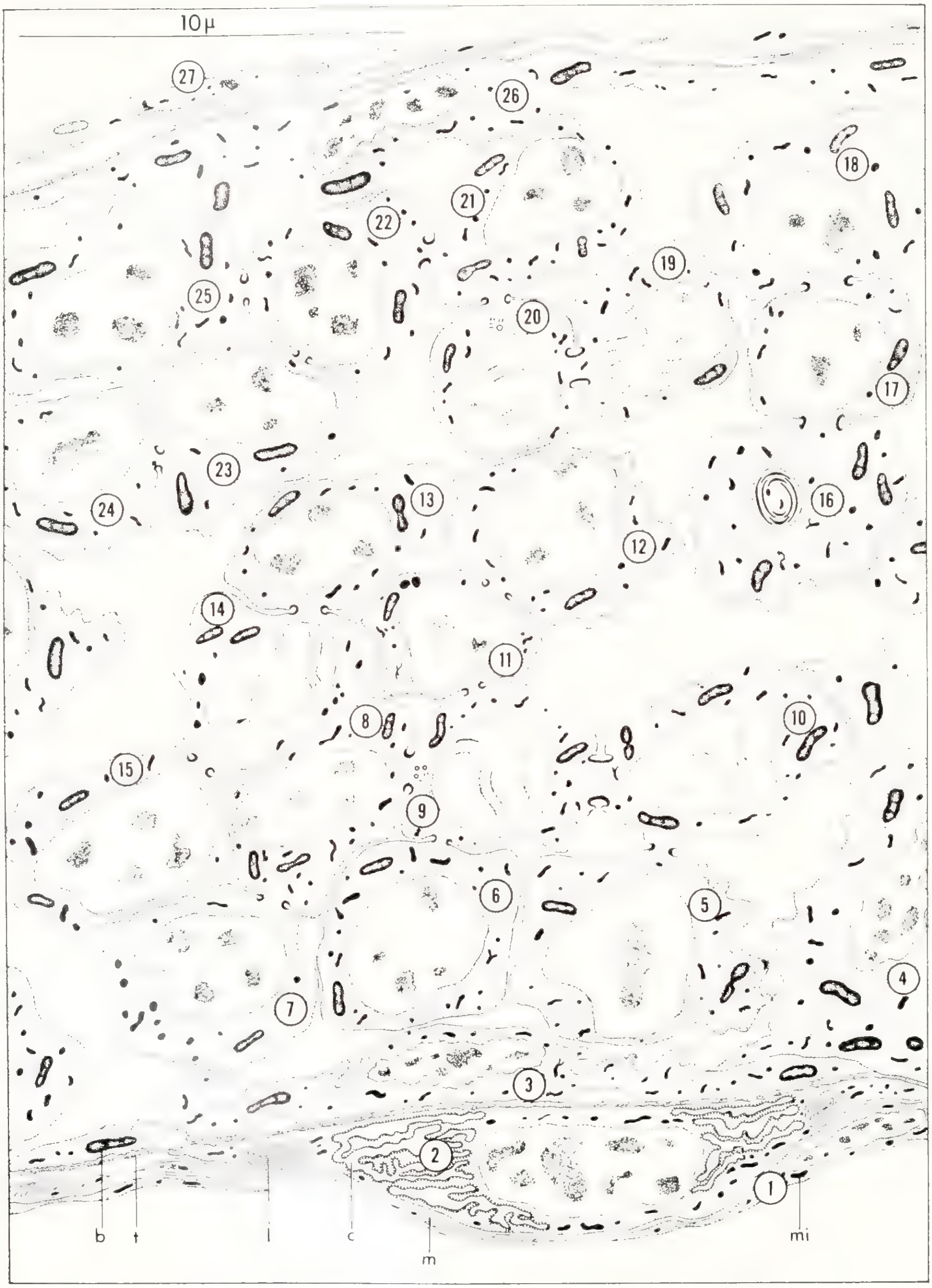
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FIGURE 2.

is 20–30 μ long and is filled with 50–100 cells characterized by large, diffusely staining nuclei (each ~4μ in diameter). We assume such cells are oogonia. The next region (G_2) is also 20–30 μ long, and it is characterized by smaller cells with more densely staining nuclei. Since clusters of metaphases are often seen in this portion of the germarium, we assume that this is the region where the cystocyte divisions occur. Since such clusters contain either 2, 4, 8 or 16 division figures, sister cystocytes are mitotically synchronized. Little order is shown in the positioning of the first, second, third, and fourth generation cystocytes in region two of the germarium, since clusters containing 2 or 4 metaphases are seen to be posterior to clusters containing 8 or 16 metaphases. The posterior portion of the germarium (G_3) is about 100 μ long. It contains post-mitotic clusters of cystocytes which are being enveloped by pro-follicle cells.

Each vitellarium in a newly eclosed wasp is composed of about 24 developing oocytes arranged in single file. Any oocyte is always in a slightly more advanced developmental stage than the one immediately behind it. There is often, but not invariably, a constriction which marks the beginning of the vitellarium. When this constriction is absent the decision as to what constitutes the first egg chamber is often quite arbitrary. The oocyte is the most posterior cystocyte in each egg chamber, and it receives a stream of cytoplasm from the anterior cluster of sister nurse cells.

In *Drosophila melanogaster* each egg chamber in the vitellarium contains an oocyte and 15 highly endopolyploid nurse cells. In *Habrobracon juglandis* each egg chamber contains a single oocyte and many nurse cells. We made careful counts of the number of nurse cells in each of 15 chambers and found the number to be 31 in each case. Therefore the cystocytes in the wasp egg chamber are *fifth* generation descendants of a cystoblast.

The nucleus of the newly formed *Habrobracon* oocyte has a diameter of approximately 4 μ. It eventually grows to a diameter of about 10 μ (Fig. 1A, oocyte 21) before it breaks down. Its DNA content presumably remains at a value equivalent to four times the haploid amount. If so, the DNA content remains constant during the time the nucleus increases in volume by a 15 fold factor. On the other hand, the nurse nuclei grow from minimum diameters of 4 μ to maximum diameters of 25 μ (a 250 fold increase in volume). In Feulgen-stained whole mounts the nurse nuclei are seen to be packed with an amorphous mass of staining threads. Since the relative intensity of stain per unit area seems about the same in nuclei of different volumes, it is clear that the DNA content of the nurse nuclei is increasing during the growth period. Obviously the nurse cell chromosomes undergo endomitotic replication, unaccompanied by polytenization. The nuclei of the nurse cells are characterized by multiple nucleoli; whereas the oocyte nucleus usually contains only one or two (Fig. 1B).

FIGURE 2. A diagram of a segment of the posterior portion of a *Habrobracon* germarium. The anterior portion of the germarium would lie to the left of the segment drawn. The organ contains loosely packed cells of various types: Pro-oocytes (cell 9, 10, 14, 19, and 20), pro-nurse cells 5–8, 11–13, 15–18, 21–25), and pro-follicle cells (cells 3, 4, 26). Cells of the epithelial sheath (1, 27) and a lumen cell (2) are also included in the diagram. Abbreviations are: b, bacterium; c, cisterna of the endoplasmic reticulum; l, lysosome; m, myofilament; mi, mitochondrion; t, tunica propria. See text for further discussion.

A monolayer of follicle cells surrounds the cystocytes. The follicle cells covering the nurse cell portion of the chamber are generally thinner than those covering the oocyte. As development proceeds the oocyte grows at the expense of the nurse cells which eventually degenerate (Fig. 1A). The mature oocyte dissected from a living ovary has a maximum diameter of about 120 μ and a maximum length of about 520 μ. Its anterior end is blunt, its posterior end is tapering, its dorsal surface is slightly concave, and its ventral surface is convex. The germinal vesicle has broken down, and the chromosomes are in metaphase of the first meiotic division. The spindle is always located anterio-dorsally; but as Speicher (1936) has pointed out, the direction of its long axis with respect to the long axis of the egg is quite variable.

The germarium and vitellarium reside within a multinucleate tube, the epithelial sheath (Figs. 1, 2). Its blind end is attached to a stalk made up of a single row of cells called the terminal filament (Fig. 1A, T) which protrudes from the germarium. The fluid-filled lumen which extends between the epithelial sheath and the ovariole surface contains small populations of "lumen cells" (Figs. 1B and 2). The posterior movement of the eggs in the ovariole is accompanied by rhythmical contractions of the epithelial sheath.

The submicroscopic morphology of the germarium

We traced composites made of overlapping electron micrographs taken at magnifications of 5000× of representative sections from sixty serials through region three of a *Habrobracon* germarium. From our subsequent reconstructions we were able to enumerate the cells in a segment about 4 μ thick and 100 μ long. A total of 160 nuclei were observed; 150 of these belonged to cystocytes, and the remainder were in peripheral "pro-follicle" cells. Twelve cystocytes contained synaptonemal complexes in their nuclei. Such cells often occurred in pairs. The center-to-center distance between their nuclei was about 4 μ, while the nuclei were themselves about 3 μ in diameter. In some cases adjacent pro-oocytes could be seen to be connected by canals. In one such pro-oocyte we were able to identify five canals, and these showed a substantial range in diameters (1.3, 1.1, 0.6, 0.5, and 0.3 μ). The widest canal connected the pro-oocyte to its sister pro-oocyte. In Figure 2 such a pro-oocyte is drawn (cell 9), but only four of the five canals are shown.

Much of the information derived from the analysis of serial sections is presented in Figure 2. Sister cells are joined by canals, each of which is surrounded by a ring-shaped rim of complex morphology. Fifteen of the canals are drawn as seen when sectioned in vertical or oblique planes, and one (that in cell 16) is shown in horizontal section. This canal is enclosed by a ring made up of eight leaves. In a previous paper (Cassidy and King, 1969) we described the detailed structure of the leaves and demonstrated that the sliding of certain leaves past one another allows such rings to dilate as the cystocytes grow.

The interconnected cystocytes can be classified as pro-oocytes or pro-nurse cells on the basis of their nuclear morphologies. The nuclei of pro-nurse cells contain multiple nucleolar masses and lack synaptonemal complexes. Each pro-oocyte nucleus contains a compact nucleolus between 0.3–0.4 μ in diameter which is made up of large particles and rodlets of unknown composition and function (see arrow, cell 14, Fig. 2). A frontally-sectioned synaptonemal complex of *Habrobracon* appears as a twisted ribbon composed of parallel lateral elements separated by a space about 0.1 μ wide. The space is occupied in turn by a medial complex which is made up of filaments that are oriented perpendicularly to the inner surfaces of the lateral elements. Approximately 70 filaments can be counted per micron of frontally-sectioned medial complex. Cystocyte nuclei are enclosed by a bilamellar envelope which is studded by annuli, each about 60 mμ in outer diameter. The center-to-center distance of adjacent annuli is about 100 mμ.

In sections of two pro-oocytes (see cells 9 and 20) we found clusters of 4 or 5 centrioles. Perhaps some of the centrioles in each cluster were carried into

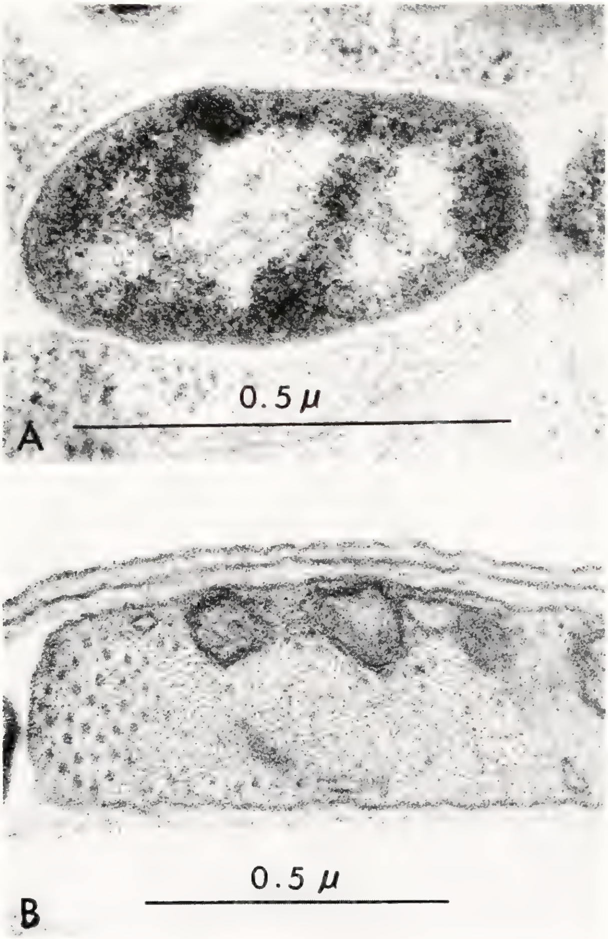

FIGURE 3 (A). An electron micrograph of a bacterium residing in a pro-oocyte in the germarium of *Habrobracon juglandis*. The micro-organism resides in a membrane enclosed space and is itself surrounded by a unit membrane. Its contents are segregated into electron dense and electron transparent areas. The former are filled with particles, the latter with fine fibrils. (B). An electron micrograph of a muscle cell from the epithelial sheath. Sectioned mitochondria and thick and thin myofilaments reside in the cytoplasm.

the pro-oocytes through the canals joining them to sister pro-nurse cells. Similar clusters of centrioles have been seen in young oocytes of *Drosophila* (Koch and King, 1969, Mahowald and Strassheim, 1970).

Flattened cells are seen at the periphery of the germarium (cells 3 and 26, Fig. 2). One gets the impression (see cell 4) that some of these cells move inward to envelope the clusters of cystocytes. This interpretation has led us to refer to them as "pro-follicle" cells. The cytoplasm of cystocytes and pro-follicle cells contains plentiful mitochondria and myriads of free ribosomes. Golgi material is scarce and the endoplasmic reticulum is undeveloped. All these cells also contain bacteria (Figs. 2, 3A). These are much larger than the mitochondria, but less plentiful. The maximum diameter and length of these microorganisms are 0.3 and 0.8 μ, respectively. They have been observed in ovaries of wasps fixed on a number of occasions, so that it is clear that they are characteristically harbored in animals, at least from the Whiting wild type stock 33.

The epithelial sheath is a multinucleate tube containing a network of muscle fibers and tracheolar cells (see cell 27, Fig. 2). Within sectioned muscle fibers one observes populations of myofilaments (Fig. 2, m; Fig. 3B) which are oriented in parallel bundles generally at right angles to the long axis of the ovariole. Two types of myofilaments are observed; one about 10 mμ in diameter and the other about 3 mμ in diameter. Presumably the thick and thin filaments contain myosin and actin, respectively (Smith, 1968, page 32). The center-to-center distance of adjacent thick filaments is about 40 mμ.

The surface of the germarium and of all developing egg chambers in the vitellarium are coated by an acellular membrane which varies in thickness between 50 and 75 mμ. The lumen cells that are often seen attached to this membrane (see cell 2, Fig. 2) are characterized by lysosomes and an extensive endoplasmic reticulum. The swollen cisternae are filled with a flocculent material of similar electron density to tunica propria.

Cystocytes grow considerably during their passage through the germarium, and each cluster is enveloped by follicle cells. Since each egg chamber in the vitellarium contains but a single oocyte, it is obvious that the other pro-oocyte must have switched to the nurse cell developmental pathway upon leaving the germarium. The oocyte and nurse cell nuclei are readily distinguishable at both the light and electron microscopic levels (Figs. 1B and 4). The border of the germinal vesicle shows characteristic invaginations not seen in the envelopes of nurse nuclei (Figs. 4, 7B). Synaptonemal complexes have not been observed in the twenty sections at our disposal, so it is likely that the medial complex has broken down by the time the oocyte enters the vitellarium. The nuclei of the nurse cells seen in electron micrographs of the earliest chambers are about 5 μ in diameter and are characterized by numerous electron dense masses which presumably represent sections through the multiple nucleoli.

Cytoplasmic transfer during vitellogenesis

Yolk is laid down in the oocyte during its passage through the vitellarium. A typical vitellogenic egg chamber is illustrated in Figure 1B. Protoplasm enters the oocyte through a funnel shaped canal. Since the minimum diameter of the canal at this stage is 5 μ, organelles in the order of magnitude of mitochondria and tubular endoplasmic reticulum can pass through it. There are only four or five nurse cells adjacent to the oocyte that empty their cytoplasm directly into the canal (see arrow, Fig. 1B). However, distal nurse cells can pass protoplasm to the oocyte indirectly by means of canals connecting them to other nurse cells which are themselves attached to the oocyte. The "nutritive appendix" which connects the oocyte to the adjacent nurse cells probably results from the fusion of the five canals which were seen to enter the germarial pro-oocyte.

The "nutritive appendix" and the areas adjacent to it are shown at higher magnification in Figure 4. The cytoplasm which is transferred from the adjacent nurse cells to the ooplasm contains mitochondria, lipid droplets, tubular endoplasmic reticulum, and myriads of free ribosomes. Membranous tubules 15 to 20 mμ in diameter and as long as 5 μ are arranged in stacks paralleling the undulating perimeter of the nurse cell nucleus (Fig. 4). This pattern of distribution suggests that membranes are synthesized at the surface of the nuclear envelope of the nurse cells, detach, and are carried along with mitochondria, lipid droplets, and ribosomes into the oocyte. The outer surface of the nuclear envelope of the nurse cells does not appear to be covered with ribosomes when this type of membrane proliferation is occurring. Ribosomes *do* adhere to the outer surface of the nuclei of follicle cells in the same chamber, and the outer nuclear membrane of nurse cells in previtellogenic stages *is* rough-surfaced. A few ribosomes are observed adhering to the cytoplasmic tubular endoplasmic reticulum.

Composites made of overlapping electron micrographs taken at magnifications of 12,500 × of 34 adjacent serial sections were traced through a region of the ooplasm adjoining the nutritive appendix. In several cases an individual strand of endoplasmic reticulum could be followed through a series of 4 to 7 adjacent sections (each 60–70 mμ). The reconstructions showed that these segments of endoplasmic reticulum (er) are no longer tubular, but are shaped like frayed ribbons. The representative dimensions of such a ribbon would be: length 3–4 μ, width 300–500 mμ, and thickness$\sim$20 mμ. At magnifications of 100,000 × or more, longitudinally or vertically sectioned ribbons can be seen to be composed of a membrane about 5 mμ thick surrounding a space about 10 mμ wide. If one compares this newly formed er with that found in adjacent follicle cells, clear cut differences are observed. There are more ribosomes per unit length of er profile in the follicle cell than in the oocyte, and the cisternae are wider and thicker in the follicle cell. Presumably these differences reflect the relative maturities of the two systems. The oocyte er is growing, whereas the follicle cell er has matured and is associated with protein synthesizing systems.

Accessory nuclei

During vitellogenesis large numbers of accessory nuclei appear in the ooplasm (Figs. 1B, 4 an). They are never seen in the nurse cells or follicle cells. Accessory nuclei are pleiomorphic structures with maximum dimensions of 2–6 μ. In sections they often display "hourglass" shapes (see Figs. 4, and 5C) which suggest that these organelles can multiply by budding. Each accessory nucleus is covered by a bilamellar envelope containing annuli that are similar in size and distribution to those seen in nuclei. The outer membrane of an accessory nucleus does not contain a coating of ribosomes. In sectioned accessory nuclei one sees

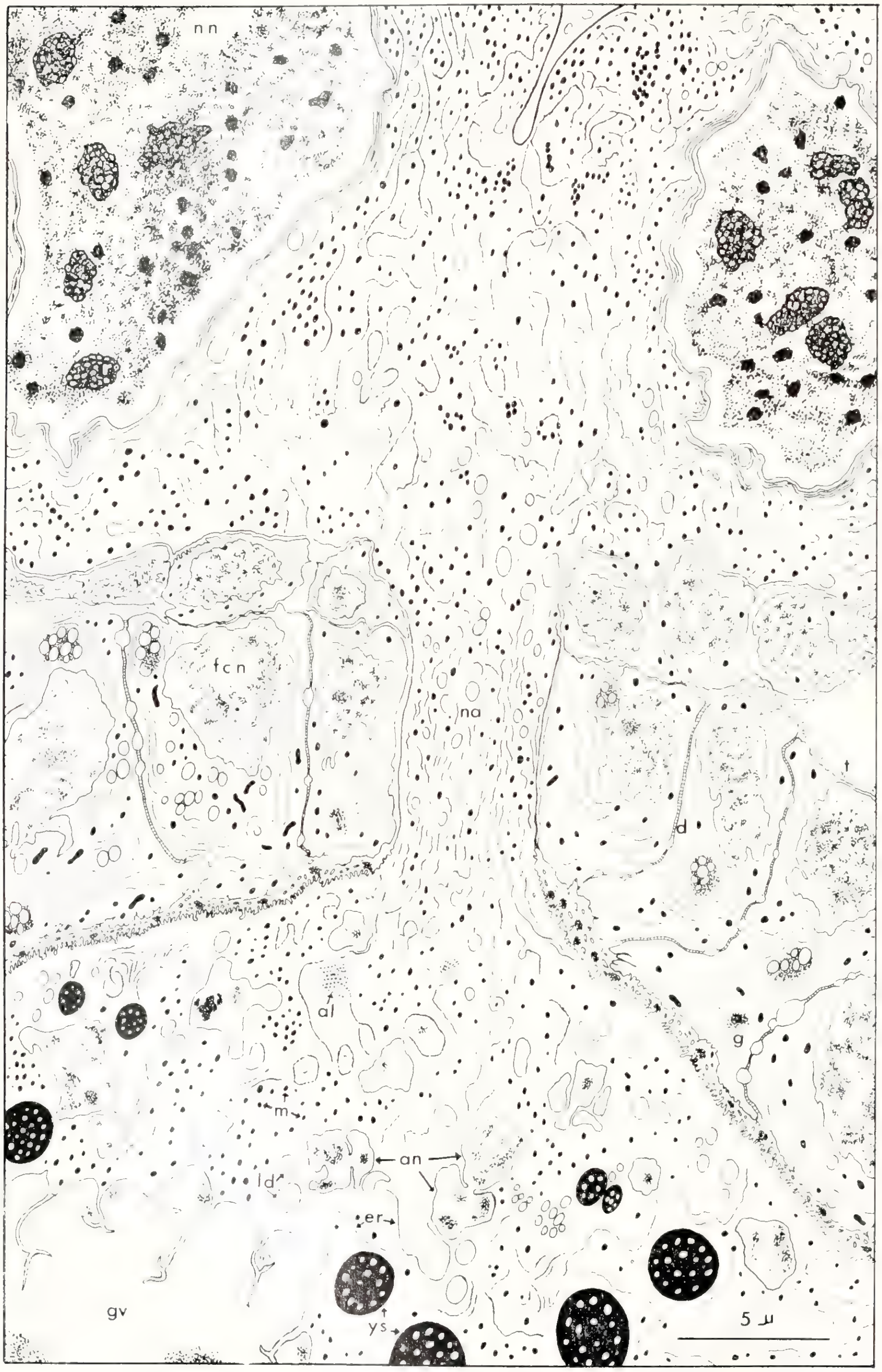
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FIGURE 4.

deposits of a variety of densities that reside in a matrix which is relatively transparent to electrons. We refer to these deposits as pseudonucleoli and subdivide them into pale, medium, and dense classes (Figs. 5A, 5C; 6C). On rare occasions annulate lamellae have been observed bordering the surface of an accessory nucleus (Fig. 5B).

In the chamber in question (number 19, Fig. 1A) accessory nuclei are concentrated in the anterior hemisphere of the oocyte, but they are not seen in the region where the nutritive appendix joins the delta of nurse cell cytoplasm entering the oocyte. Thus, it is unlikely that accessory nuclei are budded off nurse cell nuclei and transported to the oocyte. Accessory nuclei are not concentrated at the perimeter of the oocyte, and therefore there is no evidence that they bud off the oolemma or are contributed by the follicle cells to the oocyte. On the other hand, the oocyte nucleus contains nucleolar bodies which bear a striking resemblance to the pseudonucleoli of accessory nuclei (Fig. 6A-C). The pseudonucleoli of accessory nuclei and the nucleoli of the germinal vesicle and nurse cell nuclei both stain with fast green and azure B, but not with the Feulgen procedure. Therefore these bodies are composed primarily of protein and RNA.

Oocytes in whole mounts of ovarioles have been studied under phase contrast, oil immersion objectives. Accessory nuclei are not seen in the first 3 or 4 oocytes in an ovariole (Fig. 7). Each of the next 10–12 oocytes contain 2–4 large accessory nuclei in their cytoplasm. In the subsequent 4 and 5 there is a great proliferation of accessory nuclei so that hundreds are observed. These observations and those with the electron microscope suggest that the early accessory nuclei are derived from the oocyte nucleus and that they later multiply independently by budding.

We have determined the areas of each accessory nucleus detected in electron micrographs taken of the ooplasm from the 15th, 19th and 21st chambers in an ovariole. We estimate that it takes roughly one day for the 15th chamber to achieve a stage of development equivalent to that of the 21st chamber. During this time interval the average accessory nucleus increases in cross-sectional area by a factor of 3 and in volume by a factor of 5. During this same period the volume of the oocyte increases by a 70 fold factor, but the number of accessory nuclei only increases 20 times. Therefore the concentration of accessory nuclei per unit area of

FIGURE 4. A diagram of the area surrounding the nutritive appendix (na) in a vitellogenic egg chamber of *Habrobracon juglandis*. The nurse cell nuclei (nn) are gigantic and contain a complex nucleolar system made up of large numbers of small clusters of dense particles and smaller numbers of large reticulate bodies (r). Tubular membranes are stacked against the nuclear envelope. The cytoplasm of both the nurse cells and the oocyte contains myriads of lipid droplets (ld) and mitochondria (m). Accessory nuclei (an) and yolk spheres (ys) are seen only in the ooplasm, and annulate lamellae occur infrequently. The envelope of the germinal vesicle (gv) is convoluted and contains deep invaginations. The oolemma is thrown into densely packed microvilli where it borders the follicle cells. The follicle cell nuclei (fcn) are relatively small. The follicle cells bordering the oocyte are taller than those bordering the nurse cells and are characterized by whorls of endoplasmic reticulum which surround clustered lipid droplets and glycogen (g) deposits. The plasma membranes of adjacent follicle cells are joined by septate desmosomes (d). Clusters of particles of unknown composition occur in the fluid filled space between the oocyte and its follicle cell envelope. The surface of the follicle cells is covered by an acellular coating, the *tunica propria* (t). See text for further discussion.

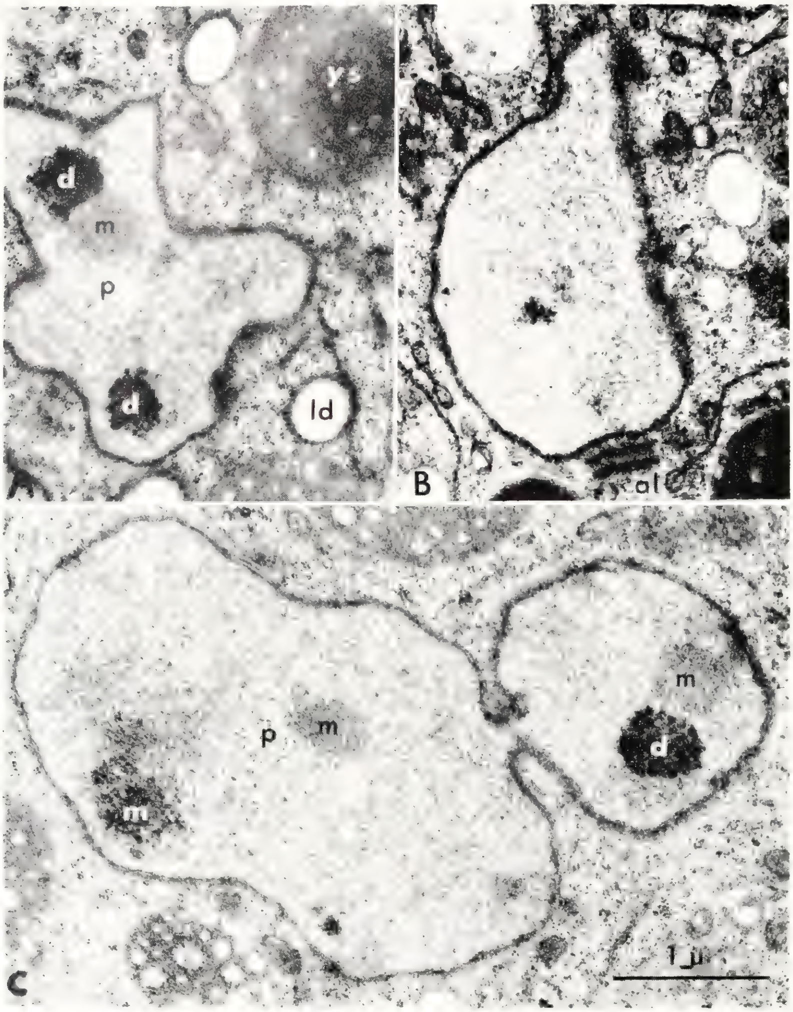

FIGURE 5. (A-C). Accessory nuclei from the ooplasm of an oocyte similar to the one shown in Figure 1B. Pseudonucleoli are characterized by different densities (p, pale; m, medium, and d, dense). Note in Figure 5C that pseudonucleoli appear in both the main body of the accessory nucleus and its bud. Abbreviations are: al, annulate lamellae; ld, lipid droplet; ys, yolk sphere.

section declines. We have been unable to detect accessory nuclei in 30 adjacent sections through a mature (metaphase I) oocyte. It follows that the accessory nuclei must degenerate during the 8–12 hours that intervene between stages represented by chambers 21 and 24 (see Fig. 1A). We interpret the accessory nucleus shown in Figure 9C, to be in the process of breaking down.

When the 21st oocyte is observed under the light microscope the accessory nuclei are seen to contain material that stains green with azure B at pH 4, green with fast green at pH 2, and is PA/S negative. We conclude that during the period that the accessory nuclei are increasing in volume they are also accumulating protein. If the new protein in the matrix is synthesized in situ, then functional ribosomes must be included among the RNP particles in pseudonucleoli. The contents of the accessory nuclei are contributed to the ooplasm about the time the ovarian oocyte enters meiotic metaphase I.

Yolk spheres

The second organelle characteristically found in the vitellogenic oocyte is the yolk sphere (Figs. 1B, 4 ys). These stain intensely with fast green (are rich in protein) and are PA/S-negative (lack significant amounts of carbohydrates). To estimate the relative abundance of different organelles in the cytoplasm of the

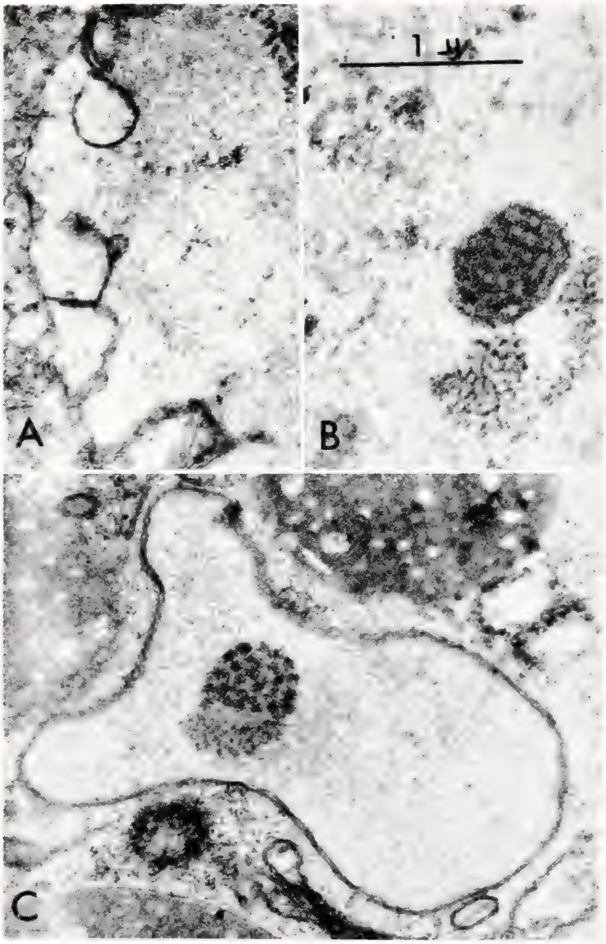

FIGURE 6. (A) The surface of the germinal vesicle showing its highly convoluted envelope. (B) Nucleolar bodies lying deeper in the same nucleus. (C) An accessory nucleus seen in the same cell. Note the similarities between its pseudonucleoli and the nucleolar bodies of the germinal vesicle.

oocyte shown diagrammatically in Figure 1B we counted the organelles in different 10 μ by 10 μ areas from a group of 20 serial sections. The areas chosen were along the left and right flanks of the delta adjacent to the oolemma and in the anterior region of the delta itself (see Fig. 1B for orientation). The lateral strip surveyed extended to a depth 80 μ from the nutritive appendix and the central strip for a distance of 30 μ. These data are summarized in Table I. Although the accessory nuclei were more abundant in the anterior region of the delta than

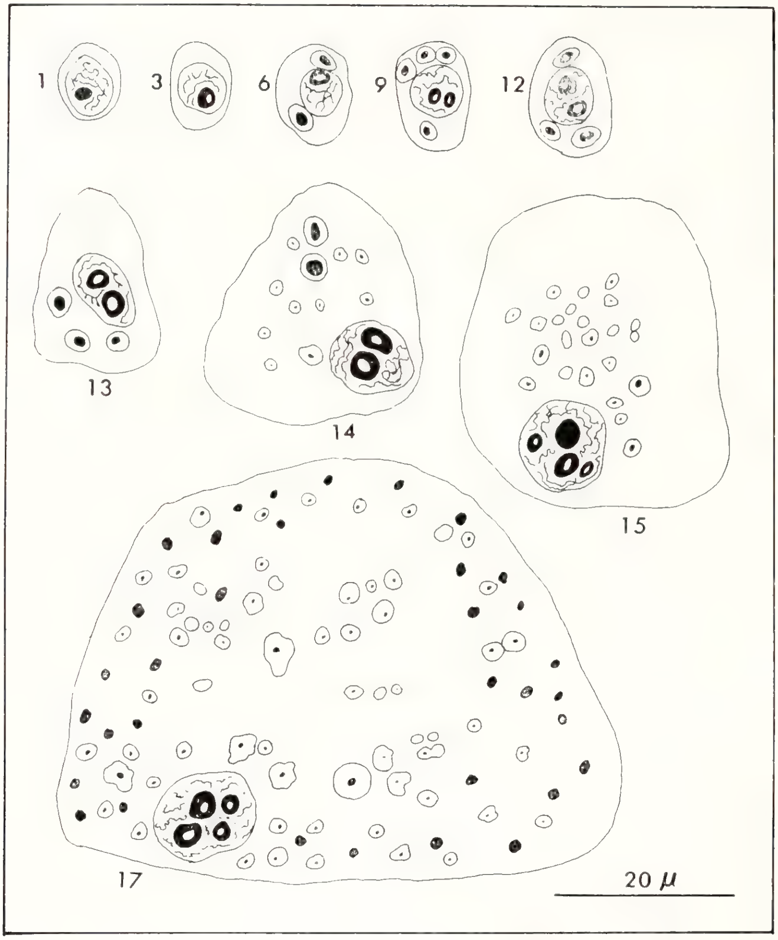

FIGURE 7. Drawings of representative oocytes from chambers at different positions in the vitellarium as seen in a Carnoy-fixed whole mount viewed under phase contrast, oil immersion optics. The nuclei of the first few oocytes contain a single nucleolus which is often ring shaped. The next 10 or so oocytes each contain 2–4 accessory nuclei, each with a prominent pseudonucleolus. Their nuclei generally contain two nucleoli. In the 14th egg chamber the ooplasm contains a few large accessory nuclei with prominent pseudonucleoli and many smaller accessory nuclei. The nuclei of oocytes 15 and 17 each contain four nucleoli. Oocyte 17 contains yolk spheres as well as accessory nuclei.

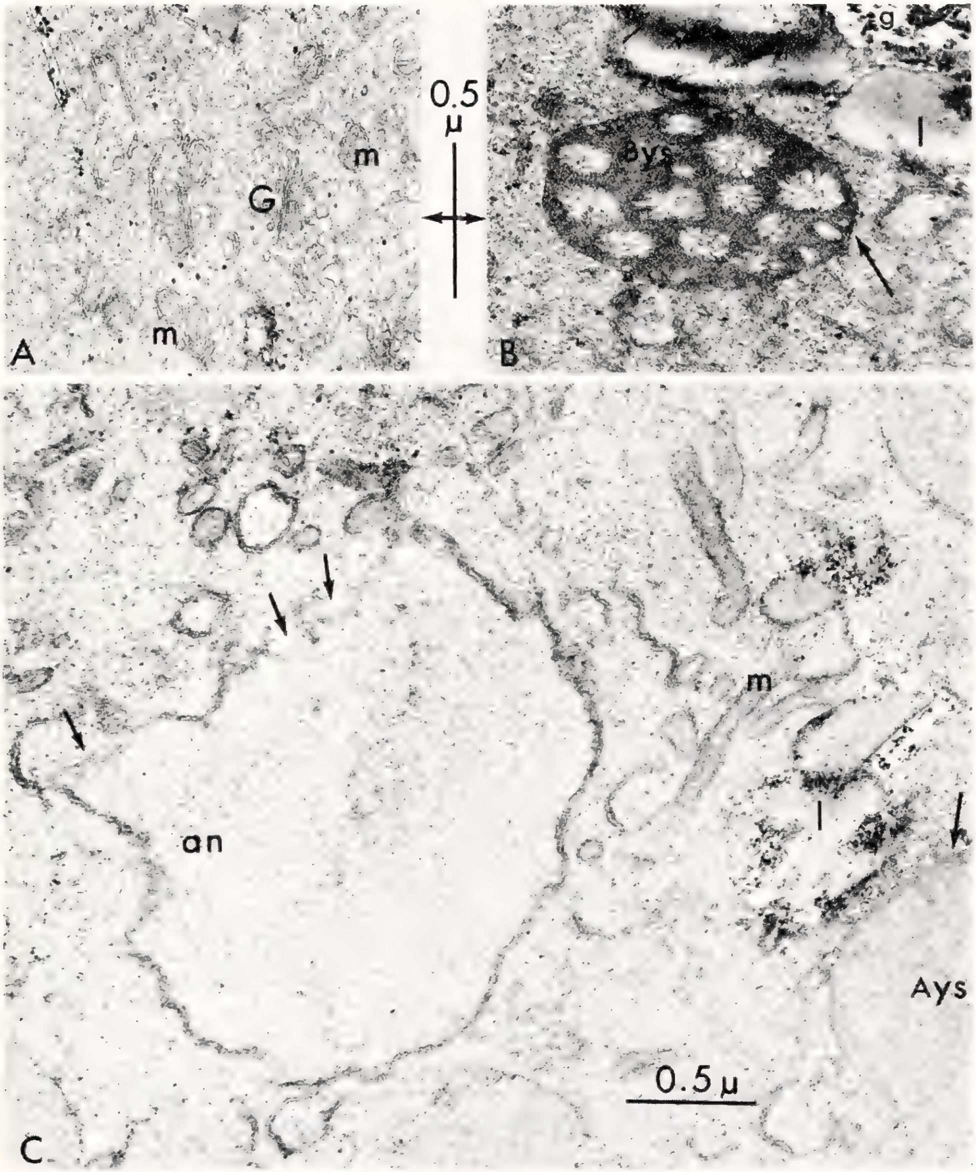

FIGURE 8. Electron micrographs of organelles seen in an oocyte equivalent to the one drawn as number 21 in Figure 1A; (A) Golgi material (G) and mitochondria (m); (B) A class B yolk sphere (Bys), lipid droplets (l), and glycogen (g); (C) The discontinuities in the envelope surrounding the accessory nucleus (an) suggest that it is beginning to break down. Arrows also point to the membranes enclosing class A and B yolk spheres.

in the left and right flanks, the yolk spheres were much more plentiful laterally than in the anterior delta. Since the concentration of accessory nuclei declines in more posterior regions of the delta, it is clear that the total number of these organelles is several times smaller than the total number of yolk spheres. However, at a slightly earlier stage yolk spheres were less common than accessory nuclei (see Fig. 7, oocyte 17). Therefore the proliferation of accessory nuclei preceeds the synthesis of yolk spheres, but once yolk sphere production is under way more organelles of this class are generated. There is no evidence that accessory nuclei are transformed into yolk spheres.

We mentioned above that we determined the cross-sectional areas of each accessory nucleus detected in electron micrographs taken of the oocytes from the 15th, 19th and 21st chambers in a *Habrobracon* ovariole. At the same time we determined the area of each yolk sphere seen in these prints. The analysis of the results indicated that the yolk spheres increased in volume an average of 2.5 times during the 24 hour developmental period studied. The total number of spheres per oocyte increased 100 times during the same interval; whereas the number of accessory nuclei only increased about 20 times.

TABLE I

Distribution of organelles in the ooplasm

The values in each column are $\bar{x} \pm s_{\bar{x}}$ (N); where
$\bar{x}$ = the mean number of organelles/100 μ^2 of ooplasm,
$s_{\bar{x}}$ = the standard error of the mean, and
N = the number of 100 μ^2 areas sampled.

	Flanks	Anterior delta
accessory nuclei	2.57 ± 0.26 (75)	5.25 ± 0.78 (16)
yolk spheres	24.71 ± 0.82 (75)	4.87 ± 0.93 (16)
lipid droplets	77.71 ± 3.68 (75)	26.44 ± 3.88 (16)
mitochondria	98.45 ± 6.21 (44)	171.29 ± 11.62 (14)

When sectioned yolk spheres are examined at magnifications of 5000 × or above they are found to fall into two classes. Class A yolk spheres contain an electron dense, homogeneous matrix; whereas in class B spheres dozens of bodies which are transparent to electrons are seen embedded in this matrix (Fig. 8). The proportions of class A and class B yolk spheres differ between the oocytes of chambers 15 and 19. Since class A yolk spheres make up about 80% of the total in the younger oocyte and only about 10% of the total in the older oocyte, we conclude that class A yolk spheres represent an earlier and class B yolk spheres a later stage in the development of the organelles. Class B yolk spheres occur throughout the ooplasm in chamber 19; whereas class A yolk spheres are restricted to a band lying within 4 μ of the oolemma. We conclude that yolk spheres arise at the perimeter of the oocyte and then move centripetally, growing and transforming from the A to the B class as they do so.

A sectioned, class B yolk sphere of 2 μ diameter contains about 60 transparent bodies, each about 0.13 μ in diameter. The total number in each yolk sphere must be in the order of 1000. Both class A and class B yolk spheres are surrounded by a single membrane (Figs. 8B and C). Yolk spheres of class C initially appear in chamber 21 and are characterized by paler matrix and a poorly defined perimeter. We assume that these spheres are breaking down and liberating their contents to the ooplasm. Since the frequency of class C yolk spheres is higher in metaphase I oocytes than in the oocyte in chamber 21, we conclude that the rate at which yolk spheres break down accelerates during the terminal stages of oogenesis.

We collected 3 hour old embryos from parasitized caterpillars and viewed them in water mounts under phase contrast optics. Next we ruptured the embryos by exerting pressure on the coverslip and were able to view the yolk spheres that were liberated into the surrounding fluid. From the deformations that the spheres underwent, it was clear that each was enclosed by a flexible membrane. As time went by, particles became visible in each sphere, and these began intense Brownian movement within the membrane. We interpreted this behavior to mean that the matrix of the spheres had liquified, and the crystals they contained now were free to engage in random Brownian movement. We assume that we were viewing class B yolk spheres and concluded, since myriads of them were present in each embryo, that they break down rather slowly.

Other ooplasmic organelles

Mitochondria are the most numerous organelles found in the ooplasm. They are continually entering the ooplasm through the nutritive appendix and are most concentrated in the anterior delta (see Table I). Lipid droplets also enter the ooplasm from the nurse cells, but in smaller numbers. Late in vitellogenesis lipid droplets appear to undergo a chemical transformation, since they develop a "coating" of PA/S-positive material. When such lipid droplets are observed under the electron microscope, they are seen to be surrounded by clusters of electron dense particles which presumably contain glycogen (Figs. 8B, C). Golgi systems are moderately abundant (Fig. 8A).

Annulate lamellae are also present in the ooplasm (Fig. 5B). However, they are quite rare ($0.03/100\ \mu^2$). Bacteria were not observed in the oocytes investigated, although $9100\ \mu^2$ of ooplasm was present in the electron micrographs scored.

Discussion

The cystocyte divisions

In *Drosophila* the cystocytes generated from a single germarial cystoblast form a *branching chain* of interconnected cells. These can be characterized by the number of ring canals each contains. Two cystocytes (designated 1e and 2e, see Koch, Smith, and King, 1967, their Fig. 7) are interconnected and each possesses 4 ring canals; two cells contain 3 canals each, four cells contain 2 canals each, and eight cells have but one canal each. In a given cystocyte cluster the canals produced at different divisions differ in morphology. It is cells 1e and 2e (the interconnected fourth generation cystocytes with the largest number of canals) that differentiate as pro-oocytes. Koch and King (1969) concluded (1) that during each of the later cystocyte divisions cells are produced which differ in that one sister receives all previously formed canals, the other none, (2) that once formed, a canal continues to increase in diameter and concurrently its rim becomes thicker, and (3) that the cue to begin construction of a synaptonemal complex is provided by the four canal rims. King and Akai (1971) suggested that the lip which surrounds each canal in a cystocyte is itself a cortical organelle derived from a contractile ring which is prevented from constricting completely by interaction with the midbody during cytokinesis. If the situation in *Habrobracon* is the same as that in *Drosophila,* except that an extra cystocyte division occurs, one would expect just what was observed, namely that a braconid pro-oocyte contains five canals, and sister pro-oocytes are interconnected by that canal with the thickest rim and greatest circumference.

In the germarial segment we investigated, pro-oocytes made up about 8% of the cystocyte population; approximately the proportion expected, if each cluster of fifth generation cystocytes contained two pro-oocytes. Since the segment we studied made up approximately one quarter of the volume contained in the posterior portion of the germarium, we estimate that there are about 20 clusters of differentiating cystocytes moving through this region at any given time. Therefore in each *Habrobracon* ovariole similar numbers of developing germ cells occur in the germarium and the vitellarium. The same is true for *Drosophila* (Koch and King, 1966).

Synaptonemal complexes

In those species with large chromosomes where a correlation is possible between light and electron microscopic observations of cells in meiotic prophase, synaptonemal complexes are first seen during zygonema, reach their maximum lengths during pachynema, and degenerate during diplonema (see King, 1970, pages 100–102 for review). Synaptonemal complexes were observed in germarial pro-oocytes, but none occurred in the nucleus of the first oocyte in the vitellarium we studied. It follows from the above observations that in *Habrobracon* the synaptic stages of meiotic prophase take place during the time that the oocyte is in region 3 of the germarium. It is presumably during this zygotene/pachytene period that crossing-over occurs (Henderson, 1970). Since the chromosomes do not enter meiotic metaphase I until the oocyte has completed development (see Fig. 1A oocytes 23 and 24), it is likely that the chromosomes in the nuclei of the vast majority of the oocytes in the vitellarium are in a diffuse diplotene stage.

Lumen cells

The fluid-filled lumen that extends between the epithelial sheath and the ovariole surface of *Habrobracon* contains small populations of amoeboid hemocytes (Figs. 1B and 2). Similar cells have been observed in the ovariole lumens of *Hyalophora cecropia* (King and Aggarwal, 1965) and *Drosophila melanogaster.* In the latter species Koch and King (1966) suggested that these hemocytes scavenge the lumen and also repair the *tunica propria* at points where it is torn during the growth and discharge of oocytes. The idea that certain hemocytes participate in the genesis of the *tunica propria* has found support in subsequent electron microsopic studies (King, Aggarwal, and Aggarwal, 1968; Beaulaton, 1968).

The epithelial sheath

Erdman (1961) has reported that the egg tube of *Habrobracon* is composed of a single epithelial layer. Since he was unable to identify muscle cells in the epithelial sheath, he concluded that developing oocytes might be transported by movements of the internal organs or of the body wall. However, we have observed muscle fibers in the epithelial sheath and find that their myofilaments are generally arranged in parallel bundles at right angles to the long axis of the ovariole. In ovarioles freshly dissected from a wasp one can observe rhythmical contractions of this sheath that result in the posterior movement of the eggs.

Orderly arranged aggregations of myofilaments are also observed in the epithelial sheath of the *Drosophila* ovary (see King, 1970, Figs. IV-5 and VI-6).

Maternal inheritance of symbiotic bacteria

Bacteria of dimensions similar to those we observed in the *Habrobracon* germarium have been described from certain strains of various *Drosophila* species (contrast Figure 3A with Figure VI-4 in King, 1970 and with Figure 8 in Kernaghan and Ehrman, 1970). In *D. melanogaster*, Wolstenholme (1965) has shown that the bacteria are noncontagious, that they are transmitted through the egg to the next host generation, and that they appear to improve the fertility of the host. Whether or not the bacteria harbored by *Habrobracon* show a maternal transmission and confer an advantage upon strains possessing them remains to be seen.

Accessory nuclei

These organelles were first described by Blochmann (1884) as bodies resembling small nuclei which occurred in developing hymenopteran oocytes. They were subsequently also observed in insects belonging to the Hemiptera, Coleoptera, Lepidoptera, and Diptera, and a controversy developed as to their origin and function (see Buchner's review, 1918). Accessory nuclei were described as arising from the oocyte nucleus, the nurse cells, or the follicular epithelium, and they were generally considered to be precursor bodies for the protein yolk spheres.

Papers published thus far showing electron micrographs of accessory nuclei are those of Hopkins (1964) for *Bombus terristris* and P. E. King and Fordy (1970) for *Ophion luteus*. In these species the accessory nuclei are covered by a double membrane possessing annulate pores. Within the accessory nuclei are inclusions which are Feulgen-negative, but stain positively for RNA. There is agreement that the accessory nuclei derive from the oocyte nucleus, but that some also arise by budding from earlier formed accessory nuclei. In neither *Bombus* nor *Ophion* was the ultimate fate of these organelles determined, but it was clear that accessory nuclei were not transformed into protein yolk spheres. Our findings in *Habrobracon* are in agreement with these earlier reports. In addition we have shown that at a relatively late stage in oogenesis (after the vitelline membrane is complete and endochorion synthesis is underway) the accessory nuclei accumulate protein and increase in volume. At the time the ovarian oocyte enters meiotic metaphase I all accessory nuclei break down and liberate their contents into the ooplasm. Thus the envelopes surrounding the accessory nuclei fragment at the same time as does the oocyte nuclear envelope.

We do not know the ultrastructural details of the origin of early accessory nuclei from the germinal vesicle. There must be a mechanism that (1) prevents any of the diplotene chromosomes in the germinal vesicle from being parcelled out among the accessory nuclei and (2) insures that the nucleic acids essential for the functioning of accessory nuclei are included in them. The observation that accessory nuclei are Feulgen-negative does not prove that they lack DNA, since this technique is relatively insensitive. The finding that accessory nuclei are capable of independent multiplication, suggests that they should contain macromolecules that are capable of replicating and transmitting information (*i.e.*, DNA). Future studies with tritiated nucleic acid precursors may elucidate these problems.

The envelopes surrounding the accessory nuclei are very similar in their ultrastructure to that of the germinal vesicle, and they all break down at approximately the same time (perhaps under the same stimulus). It seems logical to assume that the accessory nuclei synthesize proteins that are relatively unstable and are needed in the ooplasm during the rapid series of mitoses that begin shortly after the egg is laid.

Protein yolk spheres

Telfer (1954, 1960) demonstrated that in *Hyalophora cecropia* a protein that was 1000 times more abundant in the blood of female than male pupae was taken up preferentially by the ovary. On the basis of fluorescent antibody (Telfer, 1961) and autoradiographic studies (Telfer and Melius, 1963) Telfer proposed that specific blood proteins were incorporated into the oocyte by pinocytosis, and that yolk sphere formation occurred by the fusion of pinosomes. Subsequently Pan, Bell and Telfer (1969) showed that the synthesis of vitellogenic blood protein occurred in the fat body.

In *Habrobracon* there is no information available concerning the origin and fate of vitellogenic blood proteins. It is clear, however, that the protein yolk spheres originate peripherally, and the oolemma is pinocytotically active at the time these yolk spheres originate. The wasp also resembles the moth in that vitellogenesis begins during the pupal period and the completion of the vitelline membrane is delayed until very late in the vitellogenesis. On the other hand, in *Drosophila melanogaster* yolk synthesis requires the ingestion of specific nutrients by the adult (Sang and King, 1961), and the vitelline membrane is laid down long before yolk synthesis is terminated (King and Koch, 1963).

However, the yolk sphere of the fly and the wasp bear resemblance in that they undergo a similar morphological transformation as they mature. Two classes of protein yolk spheres are present in *Drosophila*. The younger (α_1) has a homogeneous internal structure and the older (α_2) contains a population of short rods embedded in a homogeneous background (see King, Bentley, and Aggarwal, 1966, Fig. 1). The reason that populations of rods appear as the protein yolk spheres mature in both species remains to be elucidated. In the case of *Drosophila* the rods are electron dense, and they exhibit a crystalline lattice having a periodicity of about 65 Å (Cohn and Brown, 1968). In *Habrobracon* the bodies found in class

B yolk spheres are transparent, and we conclude that the majority of their contents have been extracted during cytological processing.

We interpret the production of protein yolk spheres to be a mechanism for accumulating concentrated protein in thousands of packets each enclosed in a membrane. The dissolution of these organelles subsequent to oviposition supplies proteins that are hydrolyzed to polypeptides and amino acids which then serve as the raw materials for the various specific proteins synthesized by the adjacent embryonic cells.

Synthesis of rRNA by the nurse cells

Although methods for the selective extra-chromosomal replication of oocyte rDNA have evolved in some insect species (Gall, Macgregor, and Kidston, 1969), this has not taken place in the fruit fly (Mohan and Ritossa, 1970). In *Drosophila melanogaster* the chromosomes of the nurse cells undergo a series of eight or nine doublings and end up with 512 to 1024 times the haploid amounts of DNA in their nuclei (see King, 1970, Table II-1).

Since conspicuous masses of extrachromosomal DNA have not been observed in oogonia or oocytes of *Habrobracon juglandis*, we question whether amplification of rDNA occurs in this species. We know that during oogenesis the nurse nuclei increase in volume by at least a 250 fold factor, and we assume that this volume increase coincides with a cycle of 7 or 8 DNA replications. Obviously, 31 nurse cells each with an average DNA content of 256C, would multiply the rRNA cistrons available to the oocyte by thousands of times. The cistrons that transcribe the tRNAs and the mRNAs of the various ribosomal proteins would be multiplied simultaneously.

Entry of nurse nuclei into the oocyte

In his 1936 paper on *Habrobracon* oogenesis Speicher concluded that nuclei of the nurse cells do not enter the oocyte during its later stages of growth. However, according to Kaufmann, McDonald, Bernstein, Von Borstel and Das (1953) the polyploid nurse-cell nuclei disintegrate during the last stages of maturation and are engulfed by or injected into the egg cytoplasm. In *Drosophila melanogaster* Painter (1940) also claimed that the nuclear contents of the nurse cells were absorbed by the egg cytoplasm. However, in both species the canals entering the oocyte are too narrow to allow the passage of nuclei, and Feulgen-positive material has never been observed to enter healthy oocytes from the nurse cell subchamber. Herniation of nurse cell nuclei into the oocyte does occur in the case of *Drosophila* females homozygous for certain female sterile genes which affect the follicle cells that normally form a border between the oocyte and its adjacent nurse cells (King and Vanoucek, 1960).

The authors are grateful for the assistance of Mrs. Sharon Hicks, L. Beauregard, C. Bremer, and R. Marsh. The illustrations were inked by Mr. E. J. Pfiffner. This research was supported by National Science Foundation Grant GB 29279 and National Institutes of Health Biomedical Science Support Grant

5S05RR07033–05. The LKB microtome and the AEI-801 high resolution microscope were purchased with funds from National Science Foundation Science Development Grant GU2058 and the Elkhart County Cancer Society of Indiana.

SUMMARY

Normal oogenesis in the adult wasp, *Habrobracon juglandis*, is described. Accounts are given of: (1) the mitotic behavior of oogonia and cystocytes; (2) the production of synaptonemal complexes by pro-oocytes in the germarium; (3) the formation of an egg chamber and its movement through the vitellarium; (4) the ultrastructural details of the transfer of cytoplasmic organelles to the oocyte by the nurse cells; and (5) the production of accessory nuclei and protein yolk spheres in the ooplasm. Comparisons are drawn between *Habrobracon* and other insects with respect to: (1) the cystocyte divisions, the origin of ring canals, and the control of pro-oocyte differentiation; (2) the possible symbiotic relationships of bacteria which reside in ovarian tissue; (3) the proposed functions performed by accessory nuclei and protein yolk spheres; and (4) the synthesis of rRNA by the nurse cells.

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THE INORGANIC COMPOSITION OF MOLLUSCAN EXTRAPALLIAL FLUID

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The molluscan extrapallial fluid is enclosed between the inner surface of the shell and the mantle, and it is analogous to the extracellular fluid of vertebrate bone. The determination of the compositions of these skeletal extracellular fluids would give better insight into the conditions under which calcification occurs.

It has been shown that extracellular fluid of bone is separated from other extracellular fluids (Neuman, 1969), and that it has a much higher potassium concentration (Triffit, Terepka and Neuman, 1968). This fluid is very diffuse; therefore, direct analysis is difficult. Although the volume of molluscan extrapallial fluid is very small, it is more readily accessible for direct analysis.

While some studies have been made of the bone fluid, little is known of the composition of the extrapallial fluid (Wilbur, 1964). De Waele (1930), using *Anodonta cygnea*, found that the composition of the extrapallial fluid was similar to the blood. Later, Florkin and Besson (1935) demonstrated that the extrapallial fluid of this freshwater clam was a fluid compartment separated from blood.

This is a report of the inorganic composition of the extrapallial fluids of three marine bivalves: *Mercenaria mercenaria*, *Crassostrea virginica* and *Mytilus edulis*.

MATERIALS AND METHODS

The molluscs used in this study were collected from Cape Ann to Cape Cod, Massachusetts, and at Beaufort, North Carolina, throughout the year. They were maintained in aerated sea water at 21–23° and fed cultures of *Skeletonema costatum* and *Thalassiosira fluviatilis* twice weekly. (The original cultures of these diatoms were kindly furnished by Dr. R. R. L. Guillard, Woods Hole Oceanographic Institution.) Under these conditions the molluscs deposited new shell year-round as evidenced by increases of reduced weight in sea water.

Access to the extrapallial fluid was gained by drilling a hole through the center of one valve with a round dental burr. A stream of air was directed into the hole during the drilling to remove the powdered shell, and for cooling. The drilling was stopped just as the front edge of the burr penetrated the inner shell surface. A glass capillary was cemented into the hole, a short length of polyvinyl tubing fitted over the capillary, and the tubing sealed. Each animal was returned to an aquarium for at least two days before any samples were taken.

The animal was placed in a plastic dish containing two liters of Millipore (0.45 μ pore diameter) filtered sea water before the sample was taken. A sample of the sea water was taken just after sampling the extrapallial fluid. The sea water was aerated throughout this period. Samples were taken from oysters only when the valves were closed because the extrapallial cavity of this animal is in direct contact with sea water when the valves are open. Samples from the other molluscs were taken at random with respect to the opening and closing of the valves. The integrity of the mantle and the seal around the capillary was checked after the samples were taken by injecting Evans blue into the extrapallial cavity and assaying for the dye in the sea water spectrophotometrically. When any evidence of leakage was found, the samples collected for that animal were discarded. Histological examination of a number of mantles indicated that only those from which the dye leaked from the extrapallial cavity were damaged.

Total CO_2 (free and dissolved CO_2 and the salts of carbonic acid) was determined by the Conway method (Conway, 1962). These samples were taken from the proximal end of the catheter with a microliter syringe and transferred directly into the diffusion dish without being exposed to air.

The pH was determined with a combination microprobe electrode cemented into the cap of a 2-ml plastic beaker with a conical bottom. An inlet for the sample was provided by cementing a short length of capillary tubing into a hole drilled through the bottom of the beaker. This arrangement allowed a precise determination of pH on 0.1 to 0.3 ml of extrapallial fluid. Samples were taken with a tuberculin syringe fitted with a needle and then forced into the beaker.

Samples for the other analyses were taken with a microliter syringe and immediatelly diluted for these analyses. It was found that a delay of more than 10 minutes between the collection and the dilution of the extrapallial fluid samples caused them to become very cloudy. Attempts to clarify these samples by centrifugation or by pressure or vacuum filtration were only partially successful. The values obtained for calcium on the partially clarified samples were lower than on the samples diluted shortly after their collection. For these reasons, the data reported here include only those samples diluted for analyses within 5 minutes after collection.

In situ measurements of the pH of the extrapallial fluid were made in the following manner. A 5-mm hole was drilled through the shell with a trephine, a 1-cm length of 5 mm (i.d.) glass tubing was cemented over the hole, and the combination pH electrode was inserted so that the tip was even with the inner shell surface. After the extrapallial fluid filled the glass tubing, the electrode was sealed to the glass tubing with a short length of plastic tubing. The animal was then returned to sea water, and the pH of the extrapallial fluid was measured at intervals over a 6 to 12 hour period. At the end of the experiment the electrode was removed, and the integrity of the mantle was checked with Evans blue and by histological examination.

The pooled samples of mantle fluid (the fluid between the two mantles), blood plasma, and extrapallial fluid from *M. mercenaria* were obtained from 24 animals in the following manner. The mantle fluid was collected by prying the valves apart a few millimeters, and allowing the fluid to drain into a beaker. The adductor muscles were then cut, the animal blotted, and the extrapallial fluid collected by teasing the mantles away from the valves. The animal was blotted again, and the blood was collected as it drained from an incision in the foot and then centrifuged.

Calcium and magnesium were determined with a Perkin-Elmer Model 303 or a 290B atomic absorption spectrophotometer. The final dilutions of the samples and standards for Ca determination contained 1% (w/v) lanthanum chloride. Sodium and potassium were determined with a Coleman Model 12 flame photometer using a lithium internal standard. Chloride was determined with a Cotlove titrator. Sulfate was estimated by conductiometric titrations with $BaCl_2$ in 10 mN HCl.

Protein was determined by the method of Nayyar and Glick (1954) using bovine serum albumin to construct the standard curve. Hexosamine was determined according to Swann and Balazs (1962) using glucosamine as the standard. Ester sulfate was hydrolyzed by refluxing in 10 N HCl overnight. The liberated sulfate was then estimated with the barium chloranilate procedure of Wainer and Koch (1962).

RESULTS

The average inorganic compositions of the extrapallial fluids from the three molluscs are shown in Table I. The magnesium concentrations are consistently 8% greater than would be expected from standard sea water analysis (Riley and Skirrow, 1965). This overestimation resulted from the failure to include a lanthanum salt in samples (Slavin, 1968).

TABLE I

Average inorganic composition of extrapallial fluids

Animal	n	Na (mEq/l)	K (mEq/l)	Ca (mEq/l)	Mg (mEq/l)	Cl (mEq/l)	SO_4 (mEq/l)	CO_2 (mM)	pH
Mercenaria mercenaria	24*	444 ±9**	9.6 ±0.8	23.6 ±2.0	120 ±10	472 ±8	46.1 ±5.1	5.2 ±1.9	7.33 ±0.15
Crassostrea virginica	13	441 ±9	9.4 ±0.5	21.5 ±1.7	114 ±6	480 ±9	48.3 ±2.3	5.0 ±0.8	7.41 ±0.16
Mytilus edulis	10	442 ±10	9.5 ±0.5	21.3 ±1.2	116 ±6	477 ±8	47.3 ±2.3	4.2 ±0.5	7.39 ±0.17
Sea Water	47	427 ±9	9.0 ±0.1	18.5 ±0.4	106 ±5	496 ±6	51.1 ±2.6	2.5 ±0.1	7.91 ±0.11

* Number of individual determinations.
** Mean ± standard deviation.

The total cation concentration in the extrapallial fluids was always greater than the total anion concentration. This difference was due to the presence of anions, such as phosphate and succinate, which were not determined.

The Donnan ratio for each ion was calculated for each extrapallial fluid sample. Except for calcium, the Donnan ratios for the individual ions were almost identical within a species (1.05 for *M. mercenaria*, 1.03 for *C. virginica* and 1.04 for *M. edulis*). The higher values for the calcium Donnan ratios indicate that 3.2 mEq/l Ca was bound in the *M. mercenaria* extrapallial fluid, 2.0 mEq/l in *C. virginica* and 1.9 mEq/l in *M. edulis*.

The pH of the extrapallial fluid from each animal was well below that of sea water. When extrapallial fluid samples were allowed to remain in the electrode vessel up to one hour, the pH rose steadily and the samples become cloudy and viscous. After exposure to room air for one hour, the maximum pH recorded was 7.6 to 7.7, which was probably due to the loss of CO_2 from the samples. Since this pH change upon exposure to air was noted, the pH of *M. mercenaria* and *M. edulis* extrapallial fluids were measured *in situ*. The pH values obtained were within the ranges of those obtained by the sampling method. The pH of the extrapallial fluid of a mollusc decreased when the animal closed its valves and rose again when the valves were opened.

The calcium concentration in the extrapallial fluid also changed with the opening and closing of the valves. In eight specimens of *M. mercenaria*, the calcium concentration in the extrapallial fluid was 21.1 ± 1.8 (mean ± one standard deviation) and the pH was 7.41 ± 0.10 when the valves had been open for 10 minutes or longer. When the valves were closed for 15 minutes or longer, the calcium rose to 24.6 ± 2.1 mM, and the pH dropped to 7.25 ± 0.14.

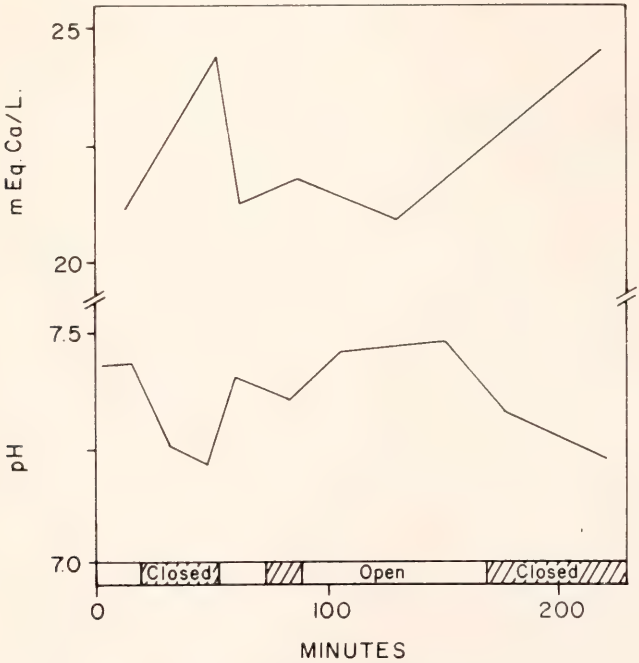

FIGURE 1. Calcium concentration and pH in the extrapallial fluid of *M. mercenaria* during the ventilation cycle. The hatched bars at the bottom of the graph indicate the periods when the valves were closed. The open bars show when they were open.

In two of the eight specimens of *M. mercenaria* and one of the six specimens of *M. edulis* examined, the minimum pH values were quite low (7.0 to 7.2). The valves of these animals remained closed throughout the period of observation. When the electrode was removed it was covered with crystalline calcium carbonate in each of these cases.

The correlation between the ventilation cycle and changes in the pH and calcium concentration in the extrapallial fluid was investigated further using *M. mercenaria*. The calcium concentration was determined in the extrapallial fluid from one side, and the pH was determined *in situ* from the other side. The data obtained from one animal are illustrated in Figure 1. The results confirmed the pre-

TABLE II

Composition of body fluids from M. mercenaria *before and after dialysis with sea water*

Fluid	Na (mEq/l)	K (mEq/l)	Ca (mEq/l)	Mg (mEq/l)	Cl (mEq/l)
	Original fluid				
Blood plasma	423	10.7	21.0	102	491
Extrapallial fluid	441	9.2	22.8	114	478
Mantle fluid	432	9.0	19.0	108	494
Sea water	427	9.0	18.5	106	496
	After dialysis				
Blood plasma	426	9.3	19.3	108	490
Extrapallial fluid	427	9.0	20.3	110	492
Mantle fluid	427	9.0	18.9	109	496

vious observations with respect to the ventilation cycle. The maximum pH observed in this series of experiments was below that observed when the extrapallial fluid samples were exposed to room air.

Calcium binding in the extrapallial fluid of *M. mercenaria* was investigated more thoroughly. In this experiment, the blood plasma and mantle fluid were compared. Table II shows the compositions of the three fluids before and after dialysis for 48 hours against 100 volumes of sea water at 4° C. The composition of the pooled extrapallial fluid was quite different from that of the pooled blood plasma, with calcium being the principal ion bound in fresh extrapallial fluid and potassium the primary ion bound in fresh blood. The mantle fluid was not different from sea water.

The Donnan ratios of the ions, except for potassium in the blood plasma and calcium in the extrapallial fluid, were reduced to unity by dialysis against sea water. This experiment demonstrated that the binding of these ions was done by a non-dialyzable fraction. The selective binding of calcium by this fraction *in vivo* appeared to be characteristic of the macromolecular components of the extrapallial fluid.

TABLE III

Composition of non-dialyzable material in the body fluids of M. mercenaria

	Impermeate* (mg/ml**)	Protein (mg/ml)	Mucopolysaccharide (mg/ml)
Blood plasma	4.25	2.14	1.27
Extrapallial fluid	3.68	0.34	3.22
Mantle fluid	1.42	0.04	0.23

* Material remaining in the dialysis tube.

** Concentration based on original volume of fluid.

Analyses of the non-dialyzable material (the impermeate) in each of these fluids were undertaken. Samples were exhaustively dialyzed against distilled water, freeze-dried, and weighed. The weights are shown in Table III.

An analysis for protein showed that 50% of the blood plasma impermeate was protein (Table III). This amount of protein could have accounted for the ionic gradients across the dialysis tubing shown in Table II. Protein accounted for less than 10% of the impermeate from extrapallial fluid.

An acid mucopolysaccharide fraction was prepared from each impermeate according to the method of Meyer, Linker, Davidson and Weismann (1953). The amounts obtained are shown in the last column of Table III. Analyses for ester sulfate and hexosamine showed that the SO_4/hexosamine ratio in the acid mucopolysaccharide fraction was 0.9 to 1.1.

Discussion

The inorganic composition of the extrapallial fluids of the three molluscs used in this study was different from that of sea water. The magnitude of the Donnan ratios for the individual ions, except for calcium, agrees with the low potentials measured across the isolated mantles of marine molluscs (C. Sterns and L. B. Kirschner, personal communications, cited by Wilbur, 1964).

In comparing extrapallial fluid and blood plasma, the potassium concentration in the extrapallial fluid was lower and the calcium concentration was higher than that of the blood plasma. Three important facts were shown by these observations: first, that the extrapallial fluid is a fluid compartment separated from blood, as found by Florkin and Besson (1935); second, that the extrapallial fluid is not formed by leakage from or damage to the cells of the mantle; and third, that the skeletal extracellular fluid of molluscs is different from that of vertebrates in this respect.

The pH values reported here were in agreement with those reported by N. Watabe and S. Kobayashi (personal communication, cited by Wilbur, 1964). The fluctuation in the pH and calcium concentration of the extrapallial fluid of an animal was associated with the ventilation cycle, with the pH falling and the calcium concentration rising when the valves closed. This is not unexpected, since the mollusc becomes anaerobic when the valves close, and previously deposited shell is dissolved to neutralize the succinic acid produced during this period (Crenshaw and Neff, 1969). Our calculations showed that a 100-g clam mobilized 2 mg shell per hour, and is large compared to the net shell formation. Thus, this turnover would mask changes in extrapallial fluid associated with shell formation, and makes estimates of shell growth using calcium-45, such as those done by Wilbur and Jodrey (1952), of questionable value.

The deposition of shell mineral on the pH electrode by some of the animals could have been normal shell formation. On the other hand, it may also have been an expression of the instability of the extrapallial fluid. However, the deposition of calcium carbonate at pH 7.0 to 7.2 was surprising.

The calcium concentration at the inner shell surface is above that of the external medium because the extrapallial fluid contains bound calcium. This binding is probably accomplished by a glycoprotein since mucopolyasccharides alone appear to have no selectivity in cation binding (Woodward and Davidson, 1968).

The bound calcium may have one of three functions. It may serve as a reservoir for calcium ions used in shell mineral formation, it may represent the dissolution of previously deposited shell (Crenshaw and Neff, 1969), or it may, with its binding agent, be a preliminary step in shell formation in which the calcium-glycoprotein complex undergoes further modification at the shell surface (Bevelander and Nakahara, 1969; Crenshaw, 1972).

I would like to thank Mrs. Jo Goulson for her aid during the preparation of this manuscript and Dr. Karl M. Wilbur for review and helpful criticism. This work was supported by USPHS Grants DE-00876 and DE-02668.

SUMMARY

The inorganic composition of the extrapallial fluids of *Mercenaria mercenaria*, *Mytilus edulis* and *Crassostrea virginica* was significantly different from sea water.

Calcium was the principal ion bound in the extrapallial fluids.

This binding was accomplished by a non-dialyzable component that appeared to be a glycoprotein.

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THE CHRONOMUTAGENIC EFFECT OF DEUTERIUM OXIDE ON THE PERIOD AND ENTRAINMENT OF A BIOLOGICAL RHYTHM

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It is thought that underlying all circadian rhythms is a "biological clock" (Brown, Hastings and Palmer, 1970), and because of the near ubiquitous distribution of clock controlled rhythms throughout the living kingdom it is important to understand completely the machinery of this living horologue. Attempts to do this have been varied, and for the most part only incompletely—if at all—successful. One approach has been to subject rhythmic organisms to specific inhibitors of macromolecular synthesis, narcotizing agents, growth stimulants, sublethal doses of general metabolic poisons, and other kinds of sustained or pulsed chemical insults, in hopes of altering the clockworks and thus gaining some insight into its mode of operation. The great majority of substances used have produced no direct alterations on the rate at which the clock runs (Bühnemann, 1955; Hastings, 1960). The only generalization that can be drawn so far is that the living clock is virtually intractable to exogenous chemical manipulation.

Some of the most interesting and paradoxical results thus far obtained are with inhibitors of protein synthesis. For example, when the dinoflagellate, *Gonyaulax* was subjected to actinomycin-D, the bioluminescent glow rhythm was inhibited. Puromycin, another inhibitor of protein synthesis, also appeared to inhibit the rhythm but in essence inhibited all bioluminescence. Chloramphenicol had no effect on the length of period, but increased the amplitude of the rhythm many fold (Karakasian and Hastings, 1962; 1963). The other rhythmic processes known in *Gonyaulax* were not affected by actinomycin D. In studying the photosynthetic rhythm in enucleated *Acetabularia*, Sweeney, Tuffi and Rubin (1967) found that other than a reduction in amplitude, actinomycin-D, puromycin, and chloramphenicol had no observable effect on this organism's clock. Some of the strongest evidence thus far obtained with inhibitors of protein synthesis lies in the effect of cyclohexamide on the *Euglena* phototactic rhythm: it was found that the period of this rhythm increased as a function of the concentration of the inhibitor (Feldman, 1967). Because the period of the rhythm was altered, it is possible that cyclohexamide may be acting directly at the level of the clock and slowing it down.

To date, the only consistent effect obtained by the application of a single substance comes from studies using heavy water. Addition of D_2O to the culture medium of *Euglena* produced both phase and period alterations in the phototactic rhythm (Bruce and Pittendrigh, 1960). Period augmentation of the bean (*Phaseolus*) sleep-movement rhythm by D_2O was found by Bünning and Baltes (1963). The activity rhythms of the deer mouse, *Peromyscus* (Suter and Rawson, 1968), the African waxbill *Estrilda* (Palmer and Dowse, 1969), the rat (Richter, 1970), and an intertidal isopod, *Excirolana* (Enright, 1971) are similarly affected. The following account describes several deuterium induced chronomutagenic (*Chrono* = time; *mutatio* = change; *genic* = producing) alterations in the spontaneous locomotor activity of the common laboratory mouse.

General Materials and Methods

The common laboratory mouse, *Mus musculus,* strain CF-1 (obtained from Carworth Farms, New City, New York) was used. This strain is known to display persistent rhythms (Dowse and Palmer, 1969). Each male was maintained in a cage (10 cm on a side) with free access to a running wheel (18 cm in diameter). Every revolution of the wheel activated a magnetic pen which recorded the event on an Esterline Angus chart recorder. Each self-cleaning cage held a 7–10 day supply of food (Purina rat chow) and H_2O or D_2O. A 99% solution of deuterium oxide, obtained from K & K Chemical Supply, was mixed with tap water to make solutions varying from 5 to 30% D_2O (in 5% increments).

The caged mice were maintained in light-proof, ventilated, sound attenuating walk-in chambers. The 20° C temperature varied adiurnally by less than 1° C. Cool-white fluorescent lamps were used as the illumination source, the light intensity being controlled by neutral density filters. Intensities were determined with a Weston model 756 photometer.

Data analysis and graphic presentation were patterned after the "array analysis" technique described in full elsewhere (Palmer, 1967). In brief, the amount of mouse activity for every hour of the day was calculated and a daily mean derived. A graph was then constructed in which each day was represented as an unshaded horizontal bar, subdivided longitudinally into 24 squares (one for each hour of the day), and all hourly activity values that equaled or surpassed the daily mean represented by blacking in the squares in the bar that corresponded to those hours. Thus represented, consecutive days are plotted one beneath the other (as in Figure 1). The net result was that minor fluctuations (*i.e.,* those below the daily mean) in the daily activity pattern were "filtered out" and do not appear on the graph, while the times of maximal activity are boldly emphasized along the otherwise unshaded bar. This method of graphic presentation condenses a great deal of data into a usable size (there are more than 96,000 mouse-hours of data used in this study) and emphasizes major trends—in particular, period estimates can be easily determined.

To quantify the effect of heavy water on the spontaneous locomotor rhythm, changes in the length of the period (measured by comparing the onsets of consecutive daily activity) were observed. The slope of lines superimposed over successive times of onset were fitted "by eye," after it was found that the "method of least squares" did not measurably improve the accuracy of this estimate (Dowse, 1971).

Experimental Rationale and Results

1. The effect of D_2O on the period of the persistent locomotor rhythm

After being subjected to a light-dark regimen of 12 hours of light (8 foot candles) alternating with 12 hours of darkness [abbreviated as LD 12:12 (8) according to the convention of Aschoff, Klotter and Wever (1965)] for 1–2 weeks, mice were switched to a constant light intensity (LL) of 0.2 foot candles for an interval

TABLE I

Period lengthening in percent of the mouse persistent locomotor activity rhythm by D_2O consumption.

D_2O	Mice in LL (0.2 ft. c.)				
	Mouse No.	Period before D_2O	Period during D_2O	Per cent difference	$\bar{X}$ difference at each dose
5%	1	24.38	24.50	.49	1.04
	2	23.00	23.30	1.30	
	3	24.25	24.57	1.32	
10%	4	24.38	24.83	1.84	2.03
	5	24.37	24.91	2.21	
15%	6	23.00	24.00	4.35	3.88
	7	23.75	24.91	4.88	
	8	24.69	25.29	2.43	
20%	9	23.38	24.86	6.33	5.67
	10	24.23	25.14	3.75	
	11	23.77	25.14	5.76	
	12	23.00	24.57	6.83	
25%	13	24.20	25.50	5.37	5.26
	14	24.25	25.50	5.15	
30%	15	23.77	25.70	8.11	7.31
	16	23.20	25.09	8.13	
	17	24.47	25.86	5.68	
	Mice in DD				
20%	18	23.17	25.14	5.76	5.67
	19	23.62	24.86	5.24	
	20	24.00	25.14	4.75	
	21	23.38	25.00	6.92	
	Blinded mice in DD				
10%	22	24.44	25.00	2.29	2.55
	23	24.43	25.00	2.33	
	24	23.75	24.47	3.03	
20%	25	24.00	25.00	4.16	5.01
	26	24.30	25.44	4.69	
	27	24.00	25.44	6.00	
	28	24.00	25.25	5.20	

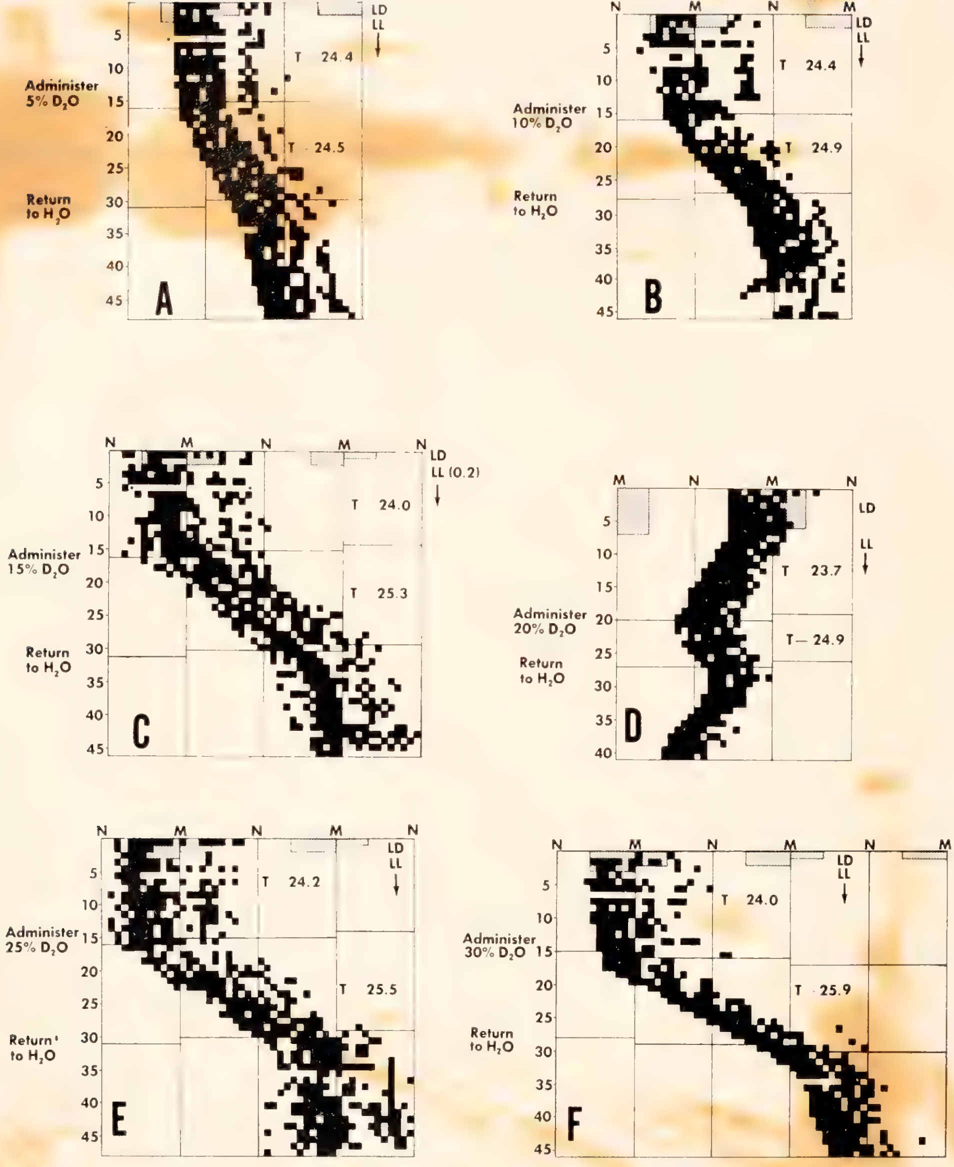
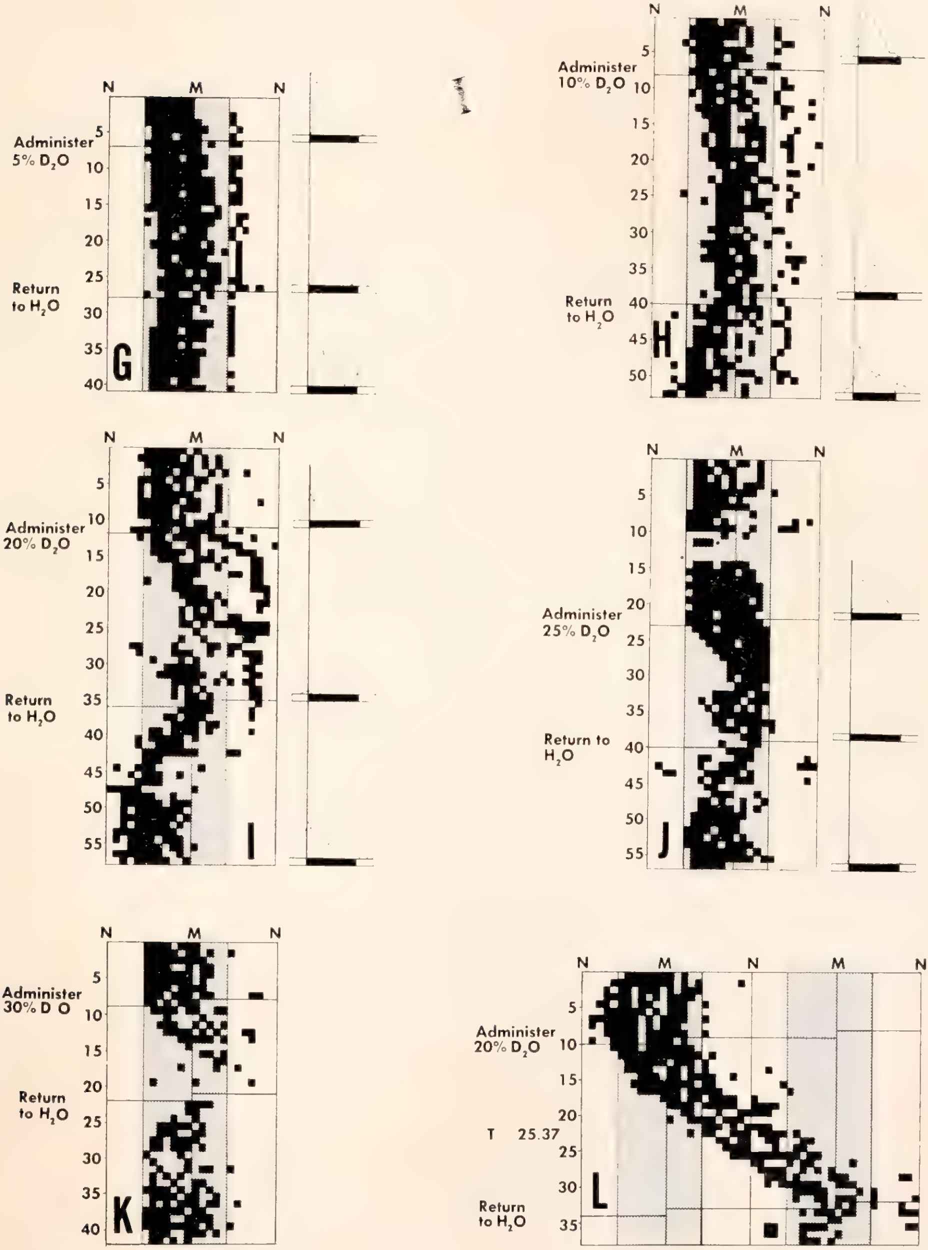


FIGURE 1. The role of D_2O consumption on the period (A-F) and phase (G-L) of the mouse circadian locomotor rhythm. 1A-F are representative examples of mice which were initially kept in LD 12:12 (8), the last few days of which are indicated by LD notations (stippling signifies times of darkness) and then switched to LL (0.2) for the remainder of the study. The intervals of D_2O ingestion, with concentrations, are indicated on right hand ordinate. T = period estimate in hours. Heavy black bands indicate times of maximum spontaneous running (described in detail in text). Note that if one tries to display circadian frequencies on a 24 hour abscissa, a time comes when the peaks will "disappear" off one side



of the graph. To prevent this, and improve graphic clarity, the abscissas are extended beyond 24 hours as necessary. 1G-K are representative examples of the degree of phase delay or period augmentation (entrainment breakaway) displayed by mice given various concentrations of D_2O (as indicated) in LD 12:12 (8). Average form-estimate curves given on right ordinate. 1L is a representative example of a mouse fed 20% D_2O in LD 12:12 (0.2).

of time (1–2 weeks) sufficient to obtain an accurate estimate of the period of their persistent locomotor rhythm. At the end of this treatment, their water bottles were substituted with ones containing D_2O in concentrations varying between 5 and 30% (in 5% increments). The mice then consumed these mixtures for the next 1–2 weeks (until a reliable period estimate could be made) and were returned to proteated water.

In all cases, D_2O increased the period of the activity rhythm, the response being a function of the deuterium concentration consumed (Table I; Figs. 1A-F and 2), with a maximum increase of about 7.4% at 30% D_2O. On return to H_2O, the period reverted to a value identical with, or close to, the pre-deuterium value, although several days were usually required—the delay presumably due to a prolonged "wash out" time. The amount of activity was also altered by D_2O ingestion [Dowse, 1971; and since confirmed by Hayes and Palmer (unpublished experiments)]. [Palmer and Goodenough (unpublished) have obtained a similar response with the waxbill, *Estrilda*].

It is known that the period of most persistent biological rhythms is a function of the intensity of the ambient constant light, *e.g.*, the mouse activity rhythm has been shown to have a period of 23.5 hours in a constant light intensity of less than 0.1 foot candle, and increases with increasing light to 25.5 hours at 20 foot candles (Aschoff, 1960). The possibility exists that deuteration may be lengthening the period of this rhythm indirectly, *i.e.*, by modifying the mouse photoreceptors or optic center so that under the influence of different concentrations of heavy water the mice "see" a constant light level as different intensities. If this is the case, one would expect the period to change—probably in a regular way—with increasing concentrations of D_2O, *i.e.*, just as we found. This possibility was examined in 2 ways: by maintaining 4 mice in constant darkness (DD) during exposure to D_2O, and to eliminate any possibility of stray or residual light in the experimental chamber, by surgically blinding 7 other mice. Bilateral enucleation was performed surgically. Diabutal was used as the anesthesia and bleeding was minimized with Gelfoam. The mice were allowed one week postoperative recovery before observing their activity patterns in the running wheels.

All 4 mice in DD were subjected to 20% D_2O for 17 days. The results (Table I) are added to Figure 2. As can be seen, these data do not differ from those obtained similarly in constant dim light.

Three blinded mice were given 10% D_2O for 13 days, while the other 4 received 20% for 16 days. These data have also been added to Table I and Figure 2. Again there is a clear lengthening of the circadian period which is completely compatible to the response of intact mice in LL.

2. *Role of deuterium on the entrainment of rhythm by LD cycles*

LD cycles are the single most effective zeitgeber known; so influential are they that the biological clock can be entrained even to artificially shortened "days," *e.g.*, 11 hours of light alternating with 11 hours of darkness. The following experiments were performed to observe the entrainability of a deuterium influenced rhythm by LD cycles.

Thirty-three mice were exposed to LD 12:12 (8) for two weeks, after which deuterium was added to their drinking water to produce concentrations varying between 5 and 30% D_2O. After observation intervals of up to 33 days, the mice were returned to proteated water again. Three classes of responses were observed: (i) no effect, (ii) a change of the phase relationship with the ambient LD cycle (always a phase delay) Figure 1H), or (iii) a "breakaway" response in which the rhythm was no longer entrained by the LD cycle (Fig. 1K). The concentration of D_2O consumed, to a large extent determined the degree of the phase delay, or the destruction of the entrainability of the LD cycle.

Figure 1G shows the data from a mouse subjected to 5% D_2O. The mouse adjusted the onset of its activity to occur 1 to 2 hours after the onset of darkness.

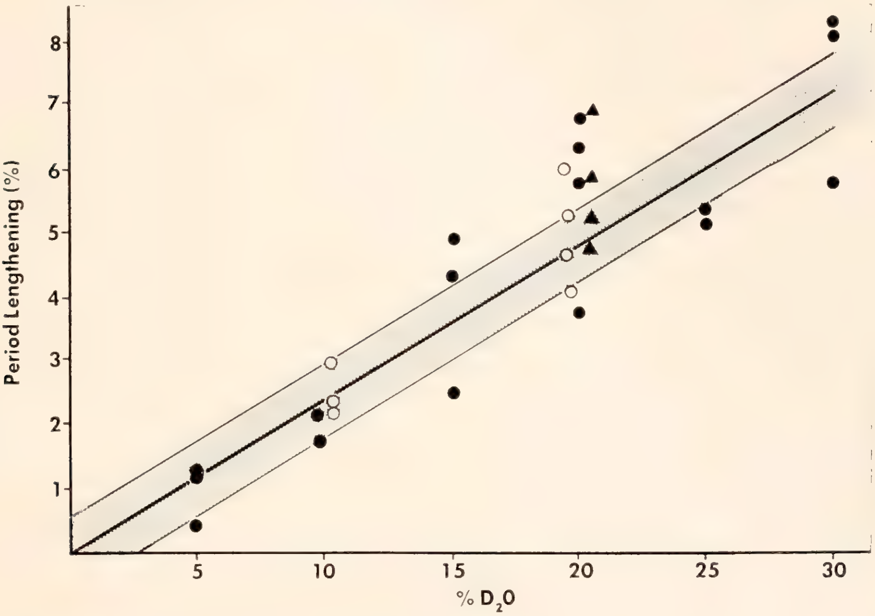


FIGURE 2. Summary of the chronomutagenic effect of D_2O on the period of the mouse circadian activity rhythm. Each point represents the percent difference between the period lengths of individual mice before and during deuterium consumption. The solid circles represent normal mice in LL (0.2), the open circles indicate blinded mice, and the triangles intact mice in DD. The curve was fitted for the mice in LL by the method of least squares (slope = 0.248; Pearson coefficient of linear correlation = 0.914; $S_y = 1.02\%$). The stippling signifies ± one standard error of the estimate.

Of the 7 mice tested at 5%, 4 delayed their phase at least 1 hour, and 3 were not affected. None broke away from the LD cycle.

The 10% dose caused a phase delay of between 2 and 3 hours in 7 of the 8 mice tested, the 8th being unaffected after a short exposure. Figure 1H shows a plot of a representative phase-delayed mouse given the 10% dose.

At 15%, 4 of the mice delayed their phase between 3 and 4 hours; one other adopted a circadian period of 25.7 hours, thus appearing to "ignore" the ambient LD cycle.

Of the 5 mice tested at 20%, one was unaffected by the treatment, 2 showed clear phase delays of about 5 hours, one broke away from the LD cycle, and one showed an unclear response. Figure 1I shows one of the phase delayed mice.

At 25% D_2O 2 mice broke away from the zeitgeber, one remained entrained with a 6-hour phase delay (Fig. 1J), and one became arrhythmic.

At 30%, 2 of the mice tested were not entrained by the LD cycle (Fig. 1K), and a third test animal became inactive at this high dosage.

In summary, the data clearly show increasing perturbations of the relationship between the LD cycle and the rhythms as the dose of deuterium becomes greater. These effects are manifested at the lower concentrations by larger and larger phase lags, and by an increase in the ratio of breakaway mice to phase delayed mice at the higher concentrations.

3. *The role of light intensity in LD entrainment of deuterated mice*

While the intensity of light used to entrain rhythms need not be particularly bright (Hastings, 1964) it has been demonstrated that increasing the intensity of light used in artificial LD cycles improves its effectiveness as an entraining agent (Wilkins, 1960). One of the clearest trends emerging from the above LD experiments is the apparent decrease in the entraining influence of LD cycles with increasing deuteration of mice. Presumably, the intensity of the LD zeitgeber should also play a role in the breakaway-or-rephase response, so the following two experiments were performed. Six mice were maintained in LD 12:12 (0.2) while a second set of 8 were exposed to LD 12:12 (80). Two of the former and 3 of the latter mice served as controls; the others were given 20% D_2O.

All four mice subjected to LD 12:12 (0.2) and 20% D_2O failed to be entrained by the ambient LD cycle (Fig. 1L). Of the 5 mice given 20% D_2O in LD 12:12 (80), all were entrained to the zeitgeber and all displayed phase delays (similar to that in Figure 1I) of about 5 hours. All control animals remained strictly entrained to their LD cycles.

These data demonstrate that increasing the intensity of light in an LD cycle produces an overriding entraining influence on the phase or period altering effect of D_2O.

Discussion

The results reported here extend the knowledge of the effects of deuterium on circadian rhythms to a new species, *Mus musculus*. Insofar as the studies parallel the one other major study done on mammals, and the few other investigations done with other organisms, the results agree quite well: the response is linear with a zero threshold. Comparing lengthening responses obtained at 30% D_2O, Suter and Rawson (1968) report a 6.45% increase, in the period of the locomotor rhythm of *Peromyscus;* Palmer and Dowse (1969) found a 6.0% lengthening of the perching activity rhythm of the waxbill, *Estrilda;* Enright (1971), a 6.33% increase in the tidal rhythm of the isopod, *Excirolana;* and (Bünning and Baltes, 1963) a 6.6% increase for the bean sleep movement rhythm. In *Mus,* the period is lengthened 7.4% by 30% D_2O ingestion.

The work presented here using blinded mice and animals maintained in DD additionally demonstrates that deuterium is not acting on the timing mechanism indirectly by altering the perception of light. It seems likely, then, that the target of deuterium lies in or very near to the clock.

Some investigators have envisioned the biological clock as one or more feedback loops, the rates of which are governed mainly by the diffusion rates of their constituents through the cell milieu. Such a model is the chronon concept (Ehret and Trucco, 1967). The period lengthening effect of heavy water certainly provides supporting evidence for such a model, as diffusion in heavy water is significantly reduced (Thomson, 1963).

Mice maintained in LD cycles and subjected to D_2O either delayed by constant intervals the phase of their locomotor rhythm, or assumed new periods, *i.e.,* appeared to ignore the ambient LD cycle and free-run. This work includes some of the first data on the effect of D_2O consumption on the entraining effectiveness of LD cycles. A preliminary report of our early findings was published in 1969 (Palmer and Dowse) and Richter (1970) has since reported in abstract, work on the hamster that supports our findings. Now, the waxbill, *Estrilda*, has also been found to respond identically (Palmer and Goodenough, unpublished experiments).

These data are in line with Aschoff's (1965) comparison of the phase relationships between organismic rhythms and LD cycles, and phase determinations of coupled physical oscillators. Using birds (Aschoff and Wever, 1962), mice (Aschoff, 1965) and lizards (Hoffman, 1968), it was shown that just as with coupled physical oscillators, the longer the period of an organism's rhythm in CC, the smaller the lead or the more the lag phase relationship when the rhythm was subjected to entraining LD cycles. In our work, we lengthened the periods of persistent rhythms with increasing concentrations of D_2O, and found that these changes manifested themselves in LD as increasing phase lags with respect to the zeitgeber cycle. As the difference between the periods of the LD cycle and rhythms was increased, a limit was eventually reached beyond which coupling was impossible; the driven rhythm then broke away and displayed its own natural frequency. In supporting Aschoff's (1965) physical-oscillator comparison, our data suggest that D_2O is acting directly at the level of the horologue complex.

Our work also shows that the increased burden placed on the zeitgeber-rhythm coupling, by increasing the natural period of the entrained rhythm with D_2O, can be counteracted by increasing the intensity of illumination during the light interval. Thus, it has been demonstrated that zeitgeber amplitude is an important factor in determining the limits within which an LD cycle can entrain a rhythm with a natural period longer than its own.

A great deal of information has been accumulated on the effects of deuterium on biological systems (for a review, see Thomson, 1963). Some of the major changes produced are: a reduction in reaction rates (*i.e.,* kinetic isotope effects), a decreased solubility of gases such as O_2 and CO_2, lowered conductivity, increased viscosity (D_2O is 18% more viscous than water at mouse body temperature), an increased acidity, a reduction in ion mobility, etc. As a result of this broad range of effects it is difficult to link a known general effect with observed rhythm perturbations.

A few possibilities suggest themselves. First, the deuterium produces an effect quite quickly: a change in period is observed within 24 hours in the waxbill and the mouse. In the isopod, Enright (1971) observed a change within a few hours after heavy water administration. Secondly, prolonged treatment does not produce larger magnitude responses, *i.e.*, the period lengthening effect is not increased with time. By combining these two observations, it can be seen that however the chronomutagenic changes are produced, they apparently do not require substantial incorporation of deuterium into organismic compounds. It seems quite likely that much of the effect may be brought about simply by deuteration of the interstitium and the aqueous protoplasm, which, among other things, would significantly alter diffusion rates. The sameness of the responses of single- and multicellular plants and animals to deuterium shows that alterations in specific organ systems, such as nervous or endocrine systems, or so-called "blood-clock barriers" (Richter, 1970), are secondary problems.

Enright (1971) has demonstrated that D_2O decreases the output of several high frequency pacemaker systems, and since each is dependent on ion exchange, he suggests that D_2O chronomutagenicity may be a result of altered ionic flux rates. This suggestion seems reasonable.

In the study of biological rhythms, the only tangible entity with which we have to work so far is the rhythm itself; the existence of an underlying control mechanism is only deduced. To date, all attempts to locate and identify this clock have failed. In addition to the clock and the overt rhythm, a third, separate entity, a *coupler,* is also involved. The evidence for the coupler derives from a variety of experiments: a single example will suffice here. A rhythm can be inhibited for a few hours, and then, when the block is removed, the rhythm will commence again in exact phase with controls (Brown *et al.*, 1970). This clearly demonstrates that the clock is not an integral part of a rhythmic process *per se*, but is coupled to it in some way, and it is via this coupling mechanism that the necessary timing information is relayed.

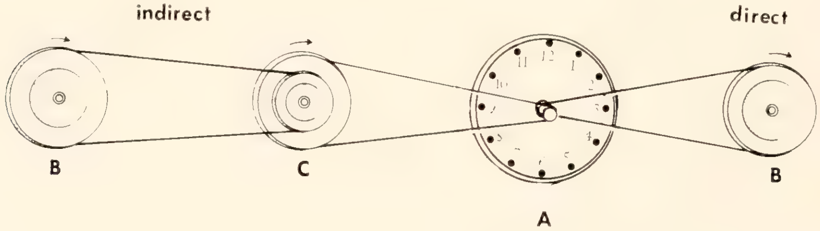


FIGURE 3. A mechanical analogy representing two means of coupling between a clock and a driven process. Details are given in the text.

Coupling may be direct or indirect; these two alternatives are represented simplistically in Figure 3, where A is the clock, B the overt driven rhythms, and C a coupler entity. The right hand paradigm illustrates direct coupling via the pulley belt, the left hand one by an interposed coupling pulley. The latter model is attractive for a number of reasons. It helps explain how an organism displaying several processes, each having a slightly different period [such as found in the oat, *Avena* (Ball and Newcomb, 1961)] could be timed by the same clock; referring to Figure 3, individual rhythms would each have its own compound coupling pulley of an appropriate size, so that each would display a different period in spite of being indirectly driven by the same clock.

Considering the possible effect of deuterium on the mechanism underlying overt rhythms in light of the above, two interpretations are equally possible, either the clock is directly slowed, or the coupling is altered. Either would account for the D_2O-induced—or other—chronomutagenic effects. If it is the coupling that is involved and it is of the direct type, the D_2O may simply cause something analogous to a belt slippage between the driving clock and the driven pulley in Figure 3. The driving pulley (*i.e.*, the clock) would continue running at an unchanged rate, but the period of rotation of the driven pulley (*i.e.*, the timed rhythm) would be lengthened. If, however, the transfer of timing information is *via* a separate coupling entity, D_2O may cause a change in this mechanism (*e.g.*, by causing a relative change in the diameters of the intermediate compound pulley in the mechanical analogy). The net result would also be an overtly different period in the rhythm based on unchanged fundamental timing information.

SUMMARY

The effect of deuterium oxide on the locomotor activity rhythm of the house mouse, *Mus musculus*, was measured in various environmental conditions.

1. In the initial experiments, in which mice were kept in constant low (0.2 foot candle) illumination, D_2O concentrations of 5% through 30% (in 5% increments) were administered via the drinking water. Increased concentrations lengthened the period, proportionally, with 30% D_2O causing a mean period lengthening of 7.4%.

2. Increasing the intensity of illumination in constant conditions can lengthen the period of the mouse locomotor activity rhythm. It was felt that deuterium might be changing the way in which the mice interpreted the light intensity, thus changing the period. Both blind mice, and mice kept in constant darkness were tested at various dosages. The results did not differ significantly from those obtained for mice in constant low illumination, thus showing D_2O does not act to alter the period by changing the way in which the mice interpret light intensity.

3. Concentrations of D_2O from 5% to 30% in 5% increments, were administered to mice in LD 12:12 (8). Doses up through 15% mostly caused phase shifts, at 20% some mice rephased and some were not entrained, while at the higher doses, the rhythms were not entrained to the LD cycle.

4. To test if increasing intensity of illumination in an LD cycle could diminish the perturbing influence of deuterium on the period and phase of rhythms, 20% D_2O was administered to mice in LD 12:12 (0.2), and LD 12:12 (80). The rhythms of all the mice in the first category were not entrained by the LD cycle, while all the mice in the second category were entrained. These data clearly indicate that the brighter the illumination during the light interval of an LD cycle, the stronger are its entraining capabilities of D_2O lengthened rhythms.

5. The results are discussed in terms of the bearing they have on clock models, and models for entrainment of rhythms to zeitgeber cycles.

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STUDIES ON *FES*, A MUTATION AFFECTING CYSTOCYTE CYTOKINESIS, IN *DROSOPHILA MELANOGASTER*

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Unlike somatic cells, the insect oocyte must contain sufficient nutrient reserves to maintain the potential organism during embryogenesis, because most insect embryos have no means for obtaining exogenous, organic raw materials. Furthermore, since little transcription of RNA takes place along DNA cistrons during mitosis, and since a period of rapid mitosis occurs early in embryogenesis, a mechanism must also exist for loading the unfertilized egg with the long-lived messenger RNAs, ribosomes, and transfer RNAs that are required to synthesize the proteins utilized during this early period of development. Higher insects have solved these problems by evolving methods for providing (1) endopolyploid cells which synthesize the required compounds and (2) a system of canals through which these products can be exported to the oocyte. In the fruitfly, *Drosophila melanogaster*, for example, the females are characterized by ovarioles that contain egg chambers in which the oocyte is one member of a cluster of 16 interconnected cells (King, 1970). The egg and the interconnected nurse cells are descendants of a single cell, the germarial "cystoblast." The interconnected cells formed by a division of a cystoblast are called "cystocytes." It is within the germarial portion of the ovariole that the consecutive mitoses occur that produce each cystocyte cluster, and it is here also that each cluster becomes enveloped by profollicle cells. The major growth of each egg chamber is completed in the more posterior portion of the ovariole (the vitellarium). Here the nurse cells undergo a series of endomitotic DNA replications and transfer their cytoplasm to the oocyte.

Females of *Drosophila melanogaster* homozygous for the autosomal recessive gene *fes* are rendered sterile because they produce "ovarian tumors" instead of eggs (see King, 1969a, for review). Each tumor in the vitellarium is composed of hundreds or thousands of cells that resemble cystocytes in that they are similar in size, are mitotically active, and are sometimes interconnected. Ovaries of *fes* genotype become "tumorous" even when they are transplanted into the abdomens of wild type females, and wild type ovaries transplanted into *fes* females do not become tumorous (King and Bodenstein, 1965). Therefore there is no evidence for diffusible tumorigenic agents as initiating factors in the development of these tumors.

Since *fes* cystocytes frequently continue dividing rather than differentiating, we concluded that the primary effect of the mutation is an alteration of the pattern of cystocyte divisions in the germarium. Normally a branched chain of sixteen cells is generated, and the characteristic pattern of connections depends on the position of the interconnecting canals. In the germarium these "ring canals" are too small to be seen with the light microscope. Therefore a *fes* germarium was reconstructed from a series of composite electron micrographs made from serial sections in order

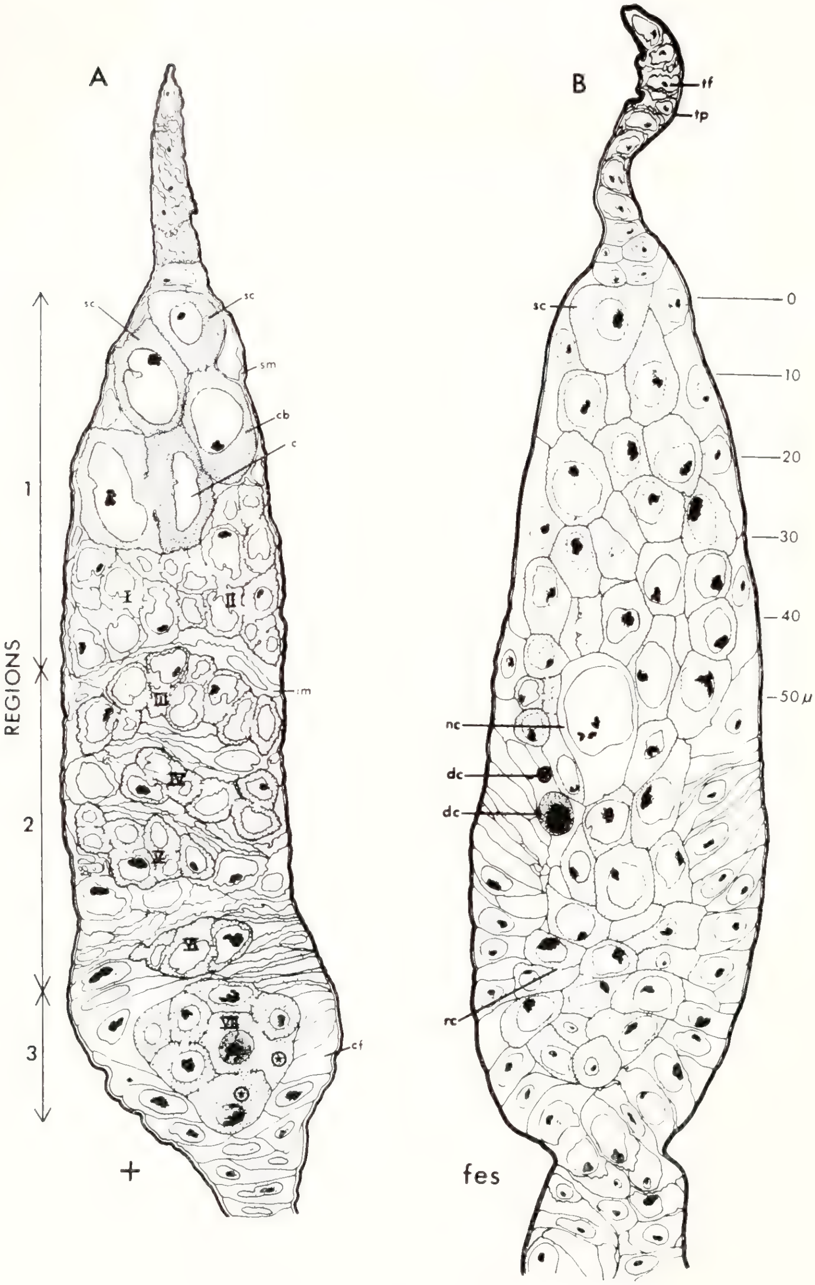
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Figure 1.

to compare the interconnections of *fes* cystocytes with the normal pattern. This reconstruction provided a detailed, but static, picture of the contents of one germarium.

In order to study the dynamics of cell division in the germarium, the mitotic figures in hundreds of *fes* and wild type germaria were observed in Feulgen-stained whole mounts. The low frequency of metaphase figures observed made it desirable to investigate germaria, in which the divisions produced over a period of several hours were accumulated. The injection of flies with the mitotic poison, colchicine, produced the desired effect. The light microscopic study provided data on the dynamics of cell division in the germarium, and it also allowed us to determine whether or not the *fes* germarium studied with the electron microscope was typical.

MATERIALS AND METHODS

Electron microscopy

Females of *Drosophila melanogaster* of genotype *S fes* + + *Alt lt*/+ *fes dp*^{tx}*Sp* + + were raised at 25° C on David's medium (David, 1962). See Lindsley and Grell (1968) for a description of the mutations used. Although the *fes* mutation has been renamed *fs(2)B* by Lindsley and Grell, the more familiar designation *fes* will be used throughout this paper. Homozygous *fes* ovaries were dissected in *Drosophila* physiological saline (the recipe is given on p. 142 of Butterworth, Bodenstein and King, 1965), and fixed for one hour at room temperature in Palade's veronal acetate buffer containing 1% osmium, and 1% potassium dichromate (Eakin and Westfall, 1962). The tissue was dehydrated through a series of aqueous ethanol solutions, transferred to propylene oxide and finally to a mixture of Epon and Araldite (Mollenhauer, 1964). Polymerization was carried out in a 60° C oven for three days.

Serial longitudinal sections were made of an entire *fes* germarium. Silver sections were cut on an LKB Ultrotome III with a section collecting side arm. The sections were mounted in groups of four on single hole grids coated with Formvar and carbon. The sections were stained for five minutes with lead citrate (Reynolds, 1963) and for fifteen minutes in saturated, aqueous uranyl acetate.

Every four section was photographed at a magnification of 4400 on a Hiatchi HS-8 electron microscope operated at 50 kV. Each of the 700 negatives obtained was enlarged 2.5 times, and the overlapping prints were assembled into series of composite electron micrographs. The cellular and nuclear membranes and the ring canals in each composite were traced on Kodak diffusion sheets. Once the tracings were stacked in order and viewed simultaneously, it became possible to follow and record the interconnections of all the cells.

FIGURE 1. (A.) A diagram of a median sagittal section through the germarium of a wild type *Drosophila melanogaster*. Roman numerals refer to sixteen cell clusters. The pro-oocytes are starred. This figure is adapted from Figure 1 of Koch and King (1966). (B.) A diagram of a median sagittal section through a germarium of a homozygous *fes* female. Mesodermal cells are drawn with pale stippling. Compare with Figure 1A, and see the text for further discussion. Abbreviations are: c, cystocyte; cb, cystoblast; cf, cuboidal follicle cell; dc, degenerating cell; im, invasive mesodermal cell; nc, nurse cell; rc, ring canal; sc, stem line oogonium; sm, squamous mesodermal cell; tf, terminal filament; tp, tunica propria.

Colchicine studies

Oregon R wild type and *fes* flies were reared for their entire life cycle at either 25° or 18° C in incubators with alternating twelve hour periods of light and darkness. Forty-eight hours after eclosion, the females were anesthetized with carbon dioxide and injected with a freshly prepared aqueous solution of colchicine (Carolina Biological Supply Co.). The injection was made using drawn-out capillaries for needles. Each injection contained 2–4 lambdas of fluid, enough to swell the abdomen slightly. Since injections of similar volumes of water had no detectable effect on mitosis, uninjected flies taken from the same cultures were used as controls. Flies were returned to the incubator in which they had been raised for the interval between injection and sacrifice.

The flies were chilled and then dissected in *Drosophila* physiological saline. The ovaries were prepared as Feulgen-stained whole mounts (see King, Burnett and Staley, 1957, page 242 for procedure). The slides were examined with a Wild M 20 research microscope at a magnification of 1250. The images of germaria were traced using a drawing tube. The drawings were calibrated with a stage micrometer. All measurements of the positions of cells or clusters were made with reference to the base of the terminal filament.

The effective concentration of colchicine for studying ovaries was determined using Oregon R females raised at 25° C. The concentration was varied from 1×10^{-2} to 1×10^{-7} M. Flies were sacrificed four hours after the injection. One hundred to one hundred fifty germaria were examined in each determination. Colchicine at a concentration of 1×10^{-4} M produced the maximum number of poisoned metaphases. A colchicine-metaphase (C-metaphase) is easily recognized because the affected chromosomes are shorter and more tightly coiled than in a normal metaphase.

In a similar study Oregon R females were injected with a 1×10^{-4} M colchicine and returned to the 25° C incubator for one to ten hours before being sacrificed. Again each determination was made utilizing one hundred to one hundred fifty germaria. Maximum values were obtained by 4 hours. Most metaphases from samples taken at 1, 2, and 3 hours were not C-metaphases; whereas all figures from $3\frac{1}{2}$ to 5 hours were C-metaphases. Later samples showed both types of metaphases. The duration of colchicine effects in cystocytes is relatively brief, but follicle cells are affected for as long as ten hours. The combination of 1×10^{-4} M colchicine, injected four hours prior to sacrificing the fly was judged optimal, and this regimen was used subsequently on Oregon R and *fes* females raised at 18° and 25° C.

RESULTS AND CONCLUSIONS

The ultrastructure of the fes *germarium*

The *fes* germarium that was reconstructed was sectioned slightly tangential to the longitudinal axis. A true longitudinal section was therefore drawn using tracings of cells seen in several composites made from the serial sections. The result, presented as Figure 1B, shows the general arrangement of the follicle cells and some of the tightly packed cystocytes. The mutant germarium should be compared to the normal germarium illustrated in Figure 1A.

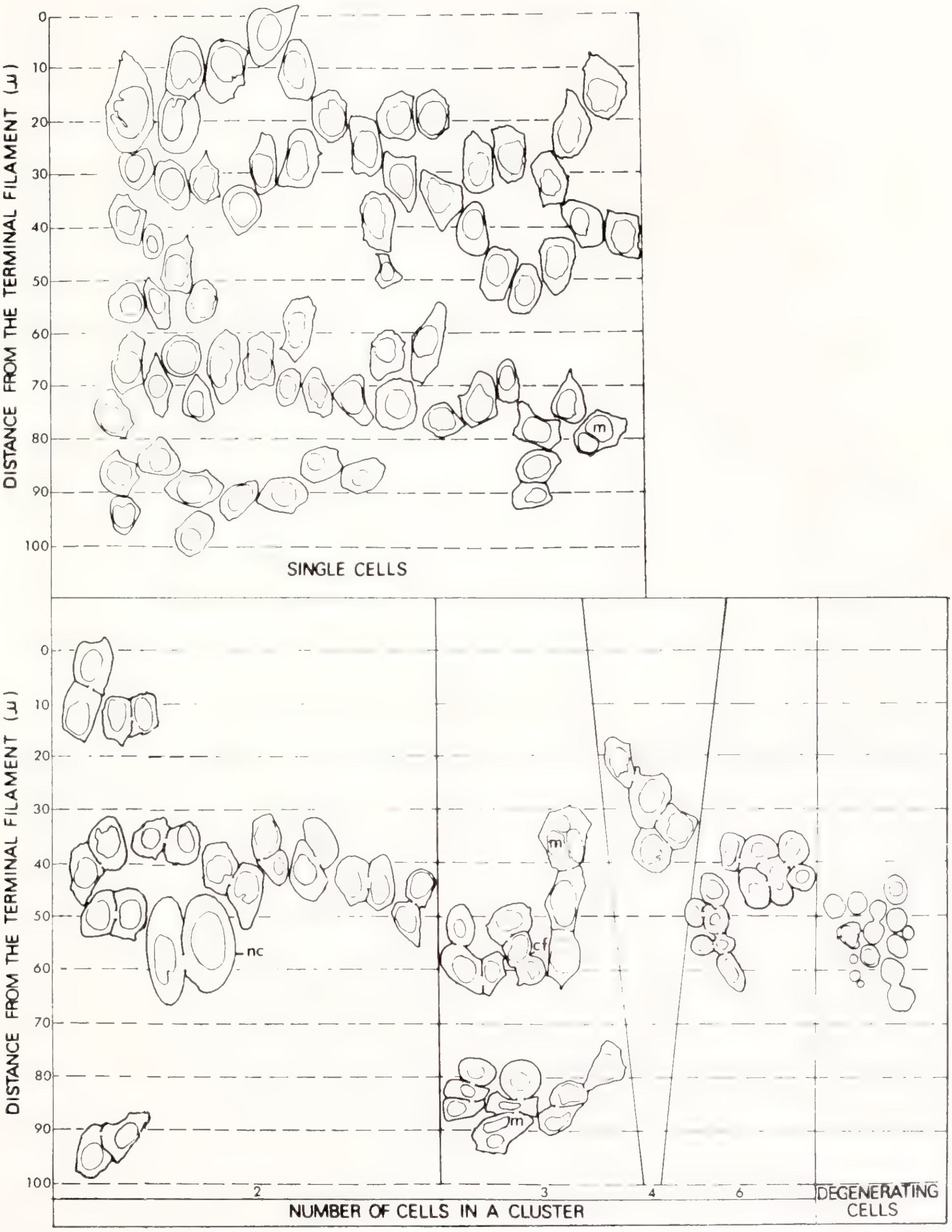
DISTANCE FROM THE TERMINAL FILAMENT (μ)
0
10
20
30
40
50
60
70
80
90
100
m
SINGLE CELLS
nc
cf
m
m
2
3
4
6
DEGENERATING CELLS
NUMBER OF CELLS IN A CLUSTER

FIGURE 2. A diagram of the contents of a *fcs* germarium based on serial electron micrographs. The cells are grouped as single cells; clusters of two, three, four, and six cells; and degenerating cells. Within each group, the cells are arranged with respect to their distance from the terminal filament. Each cell is represented by a tracing made of the electron micrograph showing the maximal cross sectional area of the cells. All ring canals are shown. Abbreviations are: cf, probable cleavage furrow; m, multinucleate cell; nc, nurse cell.

The position and interconnections of each cell in the germarium are shown in Figure 2. The maximal cross-sectional area of each cell, and all ring canals are illustrated in this diagram. The cells are arranged according to their distance from the base of the terminal filament. Individual cell volumes were estimated from the maximal cross-sectional area, using the formula $V = \pi ab^2/6$ (where "a" is the major axis and "b" the minor axis of the cross sectioned cell). The single cells in the anterior fifth were about twice the volume of those in the posterior four-fifths of the germarium. A wide range in cell volumes existed throughout the germarium. The mean volume for all of the 141 cells was 221 μ^3 with a standard error of 20 μ^3.

Fifty-four per cent of the cells in the sectioned *fes* germarium were not connected to any other cell. Such single cells were found throughout the germarium, and they sometimes contained spindle remnants, but lacked all trace of ring canals. Several unusually large single cells near the base of the terminal filament were probably stem line oogonia or cystoblasts. Most of the single cells were smaller and were similar in size to wild type cystocytes.

The remaining forty-six per cent of the cells in the *fes* germarium were in clusters containing two, three, four, or six cells. In wild type germaria, cystocyte clusters contain only two, four, eight, or sixteen cells. Clusters with fewer than sixteen cells are found only in the anterior region of the germarium, and the mean volume of the cystocytes decreases as the number per cluster increases (Koch and King, 1966). In the *fes* germarium, clusters were found in all regions, and the volume of the individual cells in a cluster did not decrease as the number of cells in the cluster increased. Two *fes* clusters with the same number of cells may contain large or small cells, and a considerable range in cystocyte sizes was also found within a single *fes* cluster.

In the wild type sixteen cell cluster, one quarter of the cells are located at branching points in the chain and have three or four ring canals. The plane of each cystocyte division is oriented so that one cystocyte retains all of the old ring canals. The hypothesis has been proposed (Koch, Smith and King, 1967, see their Fig. 7, and Koch and King, 1969) that in cystocytes both mother and daughter centrioles detach from the cell membrane, and migrate 90° in opposite directions until they are equidistant from all previously formed ring canals. Since this process is assumed to be repeated, the spindle axis is always oriented perpendicular to the spindle axis from the previous division. It appears that *fes* cystocytes have the normal ability to form such branching chains of cells (King, 1969b).

Wild type ring canal rims gradually enlarge, thicken, and accumulate on their inner surface a deposit which differs cytochemically from the rim itself (Koch and King, 1969). The rims of *fes* ring canals remain thin and delicate with little or no internal coating (Fig. 3). In the branching six cell clusters seen in the reconstructed *fes* germarium all of the canal rims had a similar appearance.

A pair of large nurse cells was found in the posterior region of this *fes* germarium (Fig. 2). Nurse cells have occasionally been observed in *fes* germaria under the light microscope (Koch and King, 1964). Migration of *fes* cystocytes through the germarium is evidently so abnormal that some cells remain there long enough to differentiate as nurse cells. A group of eleven single cells and two pairs of cells, adjacent to the nurse cells, were in various stages of degeneration. Areas of cystocyte degeneration are found occasionally in whole mounts of *both fes* and

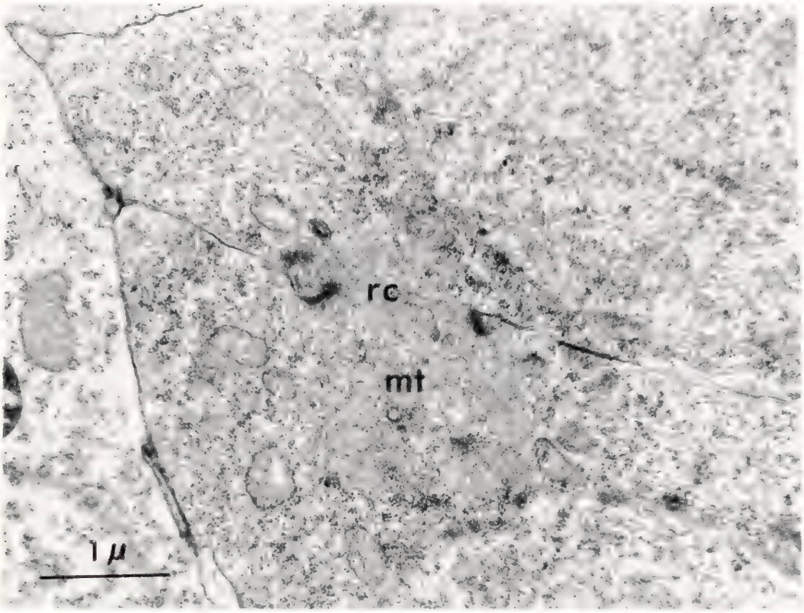

FIGURE 3. A ring canal (rc) found in the posterior half of the *fes* germarium. The canal rim has not undergone the growth and accumulation of an internal coating which are characteristic of wild type ring canals. Contrast with Koch and King (1969, their Fig. 5B). Microtubules (mt), which may represent remnants of the spindle, reside in and along side the canal.

wild type germaria, and therefore they are not a characteristic of the *fes* mutation. Cells with two and three nuclei were also found in the *fes* germarium (Fig. 2, m). According to Smith and Murphy (cited in King, 1969b), forty per cent of the cells were binucleate in *fes* chambers containing only nurse cells and oocytes.

The terminal filament, tunica propria, and the sheath of squamous cells covering the anterior region of the *fes* germarium do not differ from wild type. However, the centripetal migration of profollicle cells into the midregion of the germarium did not occur (compare Figs. 1A and B). Previous light microscopic studies of the *fes* germarium by Koch and King (1964) have shown the same general picture of a tightly packed mass of cystocytes that are not separated into regions by profollicle cells. The profollicle cells normally separate the actively dividing cells from differentiating cystocyte clusters (Koch and King, 1969).

At the posterior end of the germarium a cyst containing about fifty cells had formed. The most posterior follicle cells were cuboidal. The follicle cells at the junction of the cyst and the rest of the germarium were columnar. Although this cyst contained three times as many cells as a normal chamber, the follicle cells had not migrated between the cystocytes to form a stalk separating the cyst from the rest of the germarium. Koch and King (1964) have shown that a *fes* germarium takes about thirty hours to produce a chamber, whereas the wild type germarium takes a minimum of twelve hours. It follows that *fes* cysts separate from the germarium with great difficulty.

The reconstructed *fes* germarium was atypical in that it was not much larger than the average wild type germarium. The *fes* germaria observed in the subsequent light microscopic studies were usually about sixty per cent broader at their widest point, and contained more cells than the reconstructed germarium.

Studies on dividing germarial cells

The frequency and position of metaphase figures, and the number of simultaneously dividing cells in a cystocyte cluster were tabulated from drawings of hundreds of Feulgen-stained *fes* and wild type germaria. Most of the following data was taken from germaria pretreated with 1×10^{-4} M colchicine four hours before sacrifice. Colchicine-pretreated germaria contained four to five and a half times as many metaphases as did uninjected controls (Table I).

Stem cell and cystoblast divisions were found in the most anterior fifteen microns of wild type germaria. Approximately equal numbers of clusters of two, four and eight metaphase figures were distributed throughout the anterior thirty microns of the wild type germarium (Fig. 4). Groups with intermediate numbers of metaphases were not found. The situation in *fes* germaria was very different. Here groups with abnormal numbers of metaphases (3, 5, 6, 7, 9, 10, and 11) were observed, and the number of single metaphase and pairs of metaphases was much larger than normal (Fig. 4).

TABLE I

The average number of metaphase figures per germarium in fes *and wild type ovaries raised at 18° and 25° C*

Phenotype	Temperature	Colchicine	Germaria examined	Metaphase figures germarium
+	25° C	no	697	0.54
+	25° C	yes	475	2.35
+	18° C	no	230	0.44
+	18° C	yes	259	1.71
fes	25° C	no	244	1.19
fes	25° C	yes	221	6.35
fes	18° C	no	127	1.17
fes	18° C	yes	310	6.45

In the wild type germarium the cells in the sheath surrounding region one apparently divide infrequently, if at all, since mitoses have never been observed. Dividing profollicle cells were seen in a region about thirty microns posterior to the terminal filament. Here the profollicle cells begin to migrate between newly formed sixteen cell clusters, separating them from region one. Metaphase figures in dividing profollicle cells are more compact and oval in contour than those characterizing cystocytes.

More than twice as many dividing cells were found in the average *fes* germarium than in wild type. About half of these *fes* metaphases were seen in the posterior region of the germarium. Similar frequencies of metaphases were found in *fes* at both 25° and 18° C (Table I), whereas the frequencies of metaphases in wild type germaria were lower at the lower temperature in both the control and the colchicine

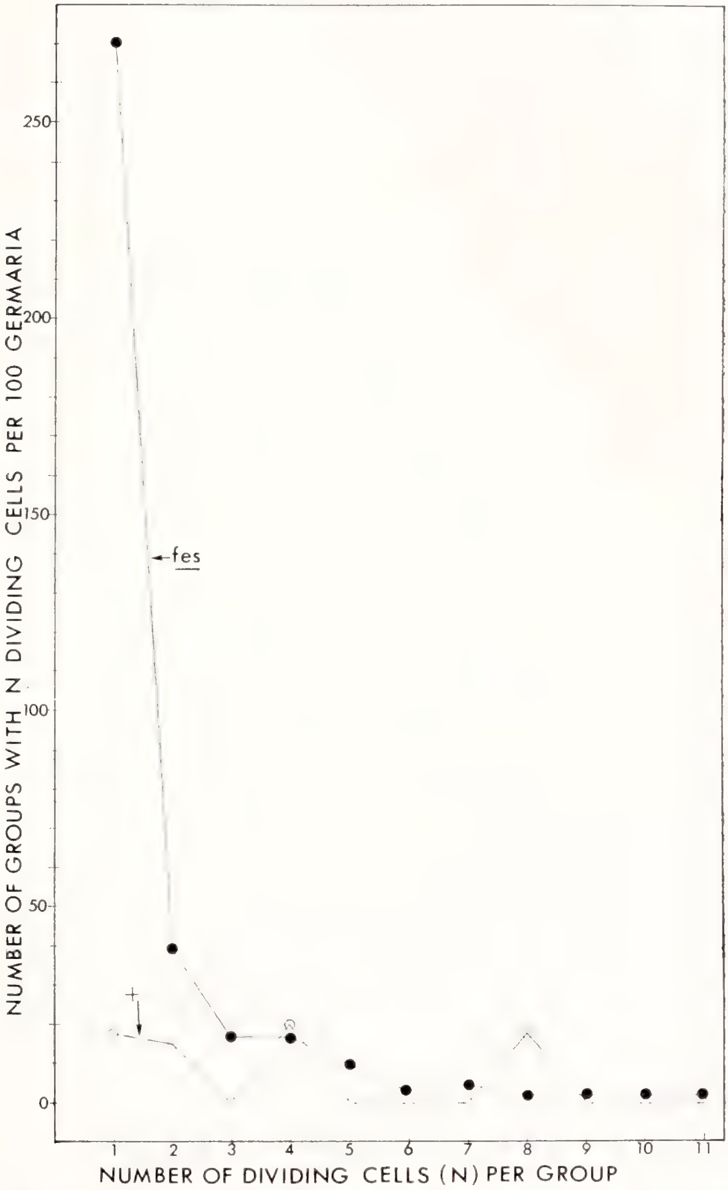

FIGURE 4. The distribution of groups with N dividing cells in germaria of colchicine-treated, wild type and *fes* females at 25° C.

series. If stem cells and cystoblasts are produced at about the same rate in *fes* and wild type, about half of the metaphases in *fes* germaria must be due to supernumerary divisions. We conclude that the average *fes* cystocyte undergoes one additional cycle of division at either temperature before leaving the germarium.

At 18° C and 25° C, thirty-eight per cent and forty-two per cent of the metaphases were in single cells. Most of the clusters contained only a few sister cells. Flies reared at 18° C had fewer single metaphases, and more large clusters. The difference between the pattern at 18° C and 25° C was significant at the 1% level using a chi square test. Single metaphases and clusters of two to eight dividing cells were found throughout the *fes* germarium. Since there are clusters with more than four dividing cells in the anterior half of the *fes* germarium, at least some clusters have undergone the normal number of divisions in this region. Ninety-two per cent of the metaphase figures in clusters of more than eight were found in the posterior half of the germarium. These clusters must have been undergoing supernumerary cycles of division.

The germaria from *fes* females are usually longer, broader, and have blunter tips than normal germaria. Geometric estimations of the volumes of both types of germaria were made, using the average dimensions from fifty drawings of whole mounts of *fes* and + germaria. The volume of the anterior five micron segment was calculated from the formula for the volume of a spherical segment [$V = \pi H^2/3\ (3R-H)$, where H is five microns, and R is the radius of the germarium five microns posterior to the base of the terminal filament]. The remainder of each *fes* or + "average" germarium was divided horizontally into a series of five micron segments. The volume of the frustrum of a right circular cone [$V = \pi H/3\ (R^2 + r^2 + Rr)$, where H is five microns; R, the radius at the larger end of the frustrum; and r, the smaller radius].

The wild type germarium has approximately one hundred twenty cells in regions one and two, excluding mesodermal cells (Smith and King, 1968). By dividing the average volume of a *fes* germarium by the average volume of one of its cells, we can estimate that there are approximately two hundred fifty cells inside of that region of the *fes* germarium equivalent to regions one plus two in the wild type germarium. The mean cell volume for the cells in the anterior fifth of the *fes* germarium was 329 μ^3, which is similar to the mean volume (311 μ^3) for cells anterior to the sixteen cell clusters in a reconstructed wild type germarium (Koch and King, 1966). Most of the cystocytes in wild type germaria are in region two, where they begin differentiation and stop growing temporarily. The cystocytes in each of these sixteen cell clusters have mean volumes of 90 μ^3 (Koch and King, 1969). Most *fes* cystocytes do not begin to differentiate after four cycles of division. Although there was a wide range in the sizes of individual cells, the mean volume for cells in the posterior four-fifths of the reconstructed *fes* germarium was 170 μ^3. This value is intermediate between the size of wild type cells in eight and sixteen cell clusters. It follows that *fes* cystocytes behave abnormally in that they continue to grow and divide in the posterior region of the germarium. As a result the average *fes* germarium contains more cystocytes than the wild type germarium, and most of these cells are larger than normal.

The anterior region of the *fes* germarium cannot be compared directly with region one of a normal germarium for two reasons. First, the actively dividing cells are not separated from older cystocytes by profollicle cells; and second, the *fes* germarium is abnormally broad and contains more cells than normal even at its anterior tip. Stem cells, cystoblasts, and cystocytes undergoing the normal number of divisions reside in the anterior region of the *fes* germarium, and it probably also contains a large number of cells that have completed the normal number of divisions.

The number of metaphase figures in each five micron segment of germaria from *fes* females was determined, and the number of metaphases in each five micron segment was divided by the volume of each segment. These data demonstrated that the frequency of division was highest in the anterior region and decreased toward the posterior region of the *fes* germarium. Thus, although *fes* cystocytes undergo supernumerary divisions, they are not capable of dividing continuously at the rate characteristic of the initial cystocyte divisions. The maximum value in the anterior region ranged from 2.7 to 2.9 metaphases per 100 μ^3 per one hundred *fes* germaria. Comparable values for region one in wild type were 3.6 at 18° C, and 4.3 at 25° C. Although the frequency of division per unit volume is less in *fes* than in wild type, this difference probably reflects the presence of "old" cystocytes that have ceased dividing in the anterior region, rather than a longer intermitotic interval for *fes*.

Since a comparison of the division rates on the basis of volume or cell number does not take into account the failure of *fes* profollicle cells to segregate the "young" cystocytes that are in the initial mitotic cycles from the mitotically inactive, "old" cystocytes, a comparison was made of the frequencies of metaphases in equal lengths of *fes* and wild type germaria. If the number of metaphases in a 30 μ long, anterior region of a wild type germarium and the number in the same length of a *fes* germarium are compared, there are 1.3 times as many divisions found in the case of *fes*. Assuming that *fes* cystocytes during their initial four cycles of division have moved no further from the apex of the germarium than has a normal cluster undergoing the same number of divisions, then there may be no difference between the initial division rates for *fes* and wild type.

The behavior of profollicle cells in the fes *germarium*

Five different classes of abnormal germaria have been described in *fes* ovaries (Koch and King, 1964). These variations from the normal germarial morphology are due primarily to abnormalities in the migration of *fes* profollicle cells. Profollicle cells may invade the posterior region of the germarium at several points without splitting off a chamber. Large tumorous masses may remain at the base of the germarium for prolonged periods of time, or tumors may separate partially from the *fes* germarium before the follicle cells have undergone a centripetal migration. The abnormal migration of follicle cells at the base of a *fes* germarium usually results in a vitellarium which contains chambers with abnormal follicular envelopes and deformed interfollicular stalks. We believe that the abnormal growth and migration of the profollicle cells in most *fes* germaria is a consequence of the abnormal clusters, rather than a direct effect of the mutation.

In wild type germaria, metaphase figures were first seen in profollicle cells in the same region where these cells begin to send protoplasmic strands between newly formed sixteen cell clusters. Profollicle cells divide infrequently, if at all, in region one. Therefore a stimulus is provided for both migration and division in region two of a normal germarium. Metaphase figures in profollicle cells were not seen in the midregion of the *fes* germaria, which usually lack centripetally migrating profollicle cells. We suggest that the centripetal migration of profollicle cells may require either the presence of a cluster containing a minimum number of cells or a change in the properties of the plasmalemma of cystocytes which coincides with the termination of their division or the beginning of their differentiation.

Metaphase figures were frequently observed in the cuboidal follicle cells in region three of the wild type germarium and at the posterior end of the *fes* germarium. As we pointed out earlier, the separation of cysts from the *fes* germarium is abnormally slow and inefficient. Apparently the organized growth of follicle cells in region three depends on the separation of clusters by profollicle cells in region two. Both the delayed formation of follicles and the continuing division of cystocytes cause the *fes* germaria to become abnormally large.

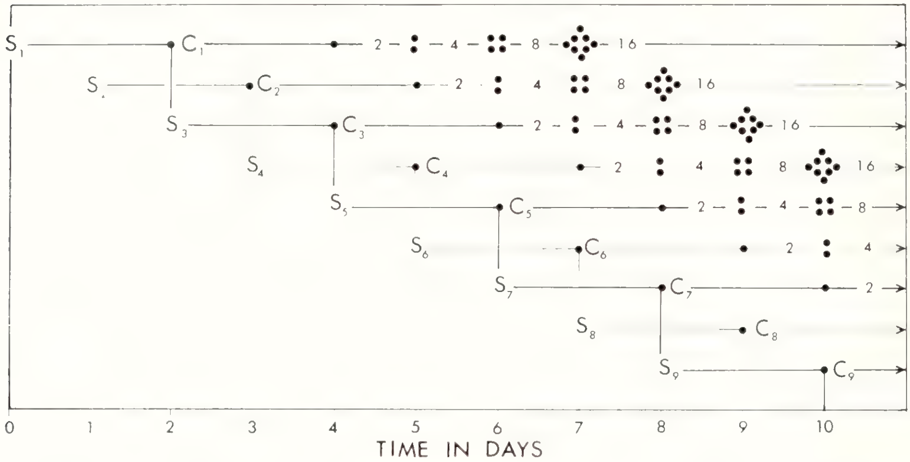

FIGURE 5. A graphic model of the functioning of a wild type ovariole. The ovariole contains two stem line oogonia (S_1 and S_2). These divide once every two days, but are out of phase by one day. Mitosis of a stem cell generates another stem cell and a cystoblast (C). This divides to produce (2) first generation cystocytes. When these divide a pair of metaphases are seen and (4) second generation cystocytes are formed. When these divide a cluster of four metaphases are seen and (8) third generation cystocytes are formed. When these divide a cluster of eight metaphases are seen and (16) fourth generation cystocysts are formed A steady state is generated in which we expect to find metaphases distributed among singles and clusters of 2, 4, and 8 in a 2:1:1:1 ratio. Stem cells, cystoblasts, and first, second, third, and fourth generation cystocytes are distributed in a 2:2:1:1:1:1 ratio.

DISCUSSION

Brown and King (1962, 1964) have presented evidence for the presence of stem line oogonia in each germarium of *Drosophila melanogaster*, and Koch and King (1966, their Fig. 2) have determined the distribution of single cells and clusters of 2, 4, 8, and 16 cystocytes in the wild type germarium. A model of the functioning of the normal germarium is presented in Figure 5. Here we assume that the fly is producing one mature egg per ovariole per day. The steady state system illustrated predicts a distribution of oogonia and cystocytes similar to that found by Koch and King, and it also predicts that the daily production of germ line metaphases should be 2 singles: 1 "twin": 1 "quadruplet": 1 "octuplet."

The experimental procedure employed in our colchicine experiments may have resulted in the collection of all division figures produced in the germaria of the injected wild type *Drosophila* during a four hour interval. The number of single metaphases and of groups of 2, 4, and 8 was about 18 per 100 germaria for each of the four categories. By multiplying by 6 and dividing by 100, we get the average value for each of the four metaphase classes, namely 1.08 per germarium per day. Since the observed value for the number of singles is one half the expected value, we conclude that the time spent during mitosis is shorter for single cells than for interconnected cells. On the other hand, the observed distribution of single cells and clusters of 2, 4, and 8 cystocytes (Koch and King, 1966) can best be explained, if one assumes that cells that double their birth size before dividing spend a longer time between divisions than do cystocytes.

The above calculations fit the expectations for a *Drosophila* female producing one egg per ovariole per day. However, it may take some time for the colchicine to reach the germarial cells in an effective concentration, and therefore the actual time during which all metaphases were collected may be considerably less than four hours. If we assume that only half of the cells, which divided during the period between colchicine injection and sacrifice of the fly were recorded, then the average value for each of the four metaphase classes would rise to 2. This would correspond to the situation where the female produced two eggs per ovariole per day, the maximum rate observed for this species (see King, 1970, page 50).

Another explanation can be put forth to account for the deficiency in the number of single metaphases found in germarial region 1. We assumed in our calculations that single cells and interconnected cells are dividing throughout the day. If single cells divide more often at night and interconnected cells divide more often during the day, then cystocyte metaphases would have been selected, since experiments were generally carried out between 10 AM and 4 PM.

One cannot argue that the deficiency in the number of single metaphases results from a difference in sensitivity of single cells and interconnected cells to colchicine, because Grell (1967, her Fig. 16) also found a 1:1:1:1 ratio of singles, twins, quadruplets, and octuplets in the ovaries of untreated pupae.

The first chamber in the vitellarium of *Drosophila melanogaster* is surrounded by an envelope containing approximately 80 cuboidal follicle cells (King and Vanoucek, 1960). These are derived from the population of profollicle cells in the germarium. Since under optimal conditions a female oviposits two eggs per day, 160 germarial profollicle cells are lost daily. It follows that the germarium must contain mitotically active, profollicle cells that serve to replenish those lost. Mitotically active, stemline oogonia reside in the anterior region of the germarium (Fig. 1A, *sc*). Our colchicine experiments demonstrate that mitotically active, profollicle cells reside in a region about 30 μ behind the terminal filament. These cells are thought to be of mesodermal origin (King, Aggarwal, and Aggarwal, 1968), and they presumably generate the cells that envelope the cystocyte cluster before it enters the vitellarium.

In *Drosophila melanogaster* the earliest step in the formation of a normal cystocyte cluster is a series of divisions which produces a branched chain of sixteen cells. We conclude that the *fes* mutation affects these divisions, since the patterns of intercellular connections and the numbers of sister cells per cluster were often abnormal in the germarium we reconstructed. The same holds true for the sister cells in tumorous and "nurse cell" chambers in the vitellarium (Koch and King, 1964). The patterns formed by the interconnected cells in the *fes* ovariole are generally asymmetric (see Koch, Smith, and King, 1967, their Fig. 9), and some of the cells normally produced during a cycle of division are missing. Therefore we conclude that the mutation affects the cystocytes in a cluster independently and at random.

Some *fes* clusters contain cells formed during the supernumerary division, and some of the cells normally found in a cluster are missing. Usually no cell with four ring canals, and consequently no oocyte, is present (King, 1969b). Clusters with more than the normal number of cells would be expected because of the high frequency of supernumerary division; however, most *fes* clusters contain very few, usually only two or three cells, and approximately 40 per cent of the germarial cells are not part of a cluster.

The abnormal patterns of interconnections found in *fes* clusters suggest that the primary effect of the *fes* mutation is the elimination of some of the cells within a cluster. This elimination occurs at random and effects any cells in a cluster independent of its neighbors, causing asymmetric deviations from the normal pattern of connections. As a consequence of this primary abnormality, the remaining cells may not stop dividing after the normal number of mitotic cycles. It is assumed that the spindle axes rotate normally, and that ring canals are stable, once they are formed. There are two simple ways in which a cell may be eliminated from a cluster. Either some cells fail to divide, or the nuclear divisions are normal, but cytokinesis is complete.

Small clusters with abnormal numbers of cells would be produced if some of the cells failed to divide in each mitotic cycle (see Fig. 6II). Although failure of cystocyte mitosis will account for the pattern of cell interconnections within a *fes* cluster, it cannot explain the action of the *fes* mutation for the reasons given below.

Normally the number of cystocystes doubles with each cycle of division. If some divisions fail to occur, the average cystocyte will produce $(2-r)$ cells and these will continue to divide forming $(2-r)^2$, $(2-r)^3$, and finally $(2-r)^4$ cells. Here r is the probability that division does not occur, and we assume the value remains constant at each cell division. According to this argument *fes* germaria should produce fewer new cells and the difference between the number of new cells generated in *fes* and wild type would be magnified with each division cycle. The *fes* germarium, however, does not show a reduced production of new cells.

All of the unconnected cells are stem cells or undivided cystoblasts according to the explanation which bases the action of the *fes* mutation on a failure of mitosis. If cystoblasts are produced even at the maximum rate of two per day, it is impossible to account for the 69 unconnected cells found in the reconstructed germarium, which was fixed 48 hours after the insect eclosed. Finally, most of the single cells in the *fes* germarium were below the volumes characteristic of either stem cells or cystoblasts. Instead the unconnected *fes* cells had the same range of volumes normally seen in cystocytes.

Normal cystocytes remain connected because cystokinesis is incomplete. A second hypothesis for the *fes* mutation is that some mutant cytoblasts and cystocytes undergo complete cytokinesis. It is assumed that the failure to form a ring canal joining sister cystocytes is a random process, which may independently effect any dividing cell during any cycle of division. Since cytokinesis, rather than nuclear division, is abnormal, the total number of cells would double in each cycle

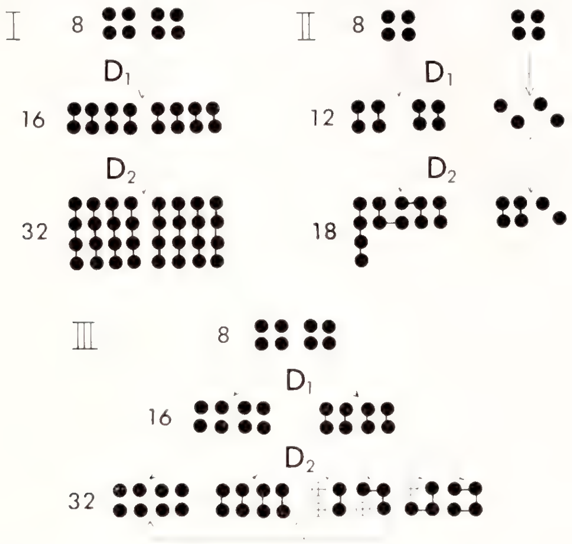

FIGURE 6. A diagrammatic comparison of the first two cycles of cystocyte division in wild type germaria (I) and in mutant germaria (II and III). In mutant model II the division of half of the cystocytes is suppressed, but those that do undergo mitosis, form canals in the normal way. In mutant model III all cystocytes undergo mitosis, but half of the cells undergo complete cleavage and the other half form ring canals during each division cycle. D_1 and D_2 represent the first and second division cycles. Each diagram begins with eight cystoblasts. (I) In the wild type germarium each cystoblast divides once forming a two cell cluster. Both cells in each cluster divide a second time producing a chain of four cells connected by ring canals. The final number of cells is 32. (II) In the mutant germarium half of the cells fail to multiply in each cycle, and the 2 unconnected cells present in the final population are those cystoblasts which failed to multiply on both occasions. Clusters of three or four interconnected cells are formed, if one or both of the cells in a two cell cluster divided during the second cycle. Although this scheme produces the small, abnormal clusters found in *fes* germaria, it cannot generate large numbers of unconnected cells. Note that the total number of cells after each division cycle is less than in wild type. (III) In this model half of all cystocyte mitoses in the mutant are followed by complete cytokinesis. Cystoblasts form either a two cell cluster, or two unconnected daughter cells. These unconnected daughter cells may in turn form two cell clusters, or additional unconnected cells. If one or both of the cells in a two cell cluster forms a ring canal during the next division, a three or a four cell cluster is made. As in wild type (I), the total number of cells doubles during each division cycle. Unlike model II, unconnected daughter cells are generated from joined cystocyte pairs during D_2. These single cells are symbolized by ⊕, and they join the pool of single cells generated by cells that were never part of a cluster. Since some unconnected cells may start new clusters, a single cystoblast may eventually give rise to more than one cluster. In the example shown, 8 cystoblasts produce (in two cycles of division): 1 four-cell cluster, 2 three-cell clusters, 5 two-cell clusters, and twelve unconnected cells. During the second cycle of division some cells are budded off clusters, and these single cells can be treated mathematically in two different ways. In the algebraic model in Figure 7A we assume that they continue to multiply, but only a given fraction of the daughter cells remain connected. In Figure 7B we assume the cells continue further multiplication, but daughter cells invariably undergo complete cleavage.

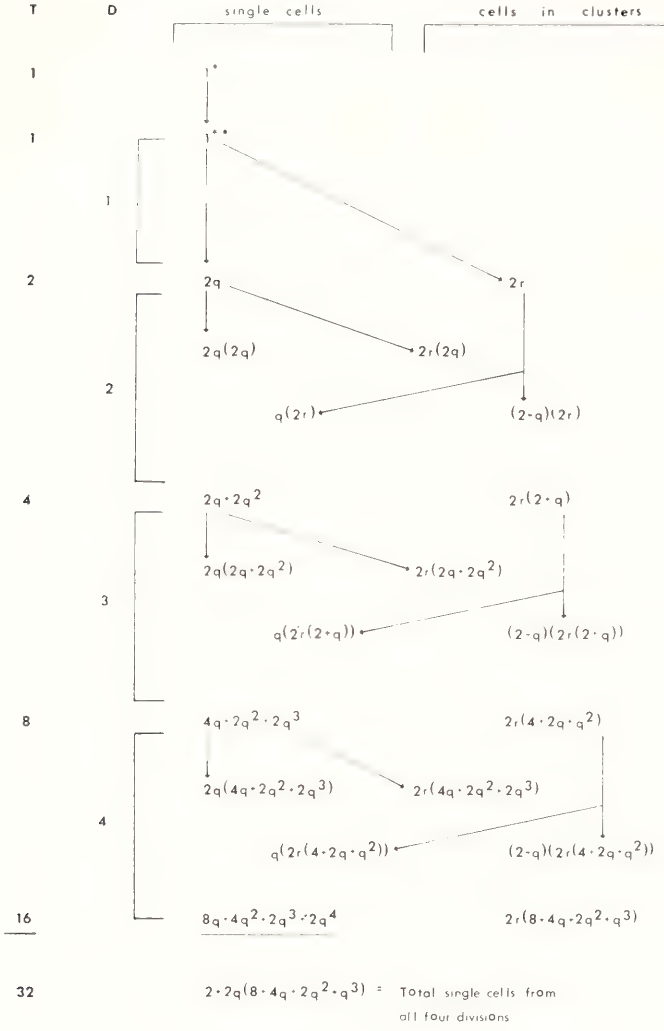
T
D
single cells
cells in clusters
1
1*
1
1**
1
2
2q
2r
2q(2q)
2r(2q)
2
q(2r)
(2-q)(2r)
4
2q·2q²
2r(2·q)
2q(2q·2q²)
2r(2q·2q²)
3
q(2r(2+q))
(2-q)(2r(2·q))
8
4q·2q²·2q³
2r(4·2q·q²)
2q(4q·2q²·2q³)
2r(4q·2q²·2q³)
4
q(2r(4·2q·q²))
(2-q)(2r(4·2q·q²))
16
8q·4q²·2q³·2q⁴
2r(8·4q·2q²·q³)
32
2·2q(8·4q·2q²·q³) = Total single cells from all four divisions

FIGURE 7A

FIGURE 7. A pair of algebraic models which generate the probable frequencies of single cells and of cells in clusters, if a constant fraction of cells undergo complete cytokinesis in each division cycle. Here q is the probability that a connected cell will undergo complete cytokinesis. r is 1-q, the probability that a cell will form a ring canal. 1* represents the stem line oogonium. 1** represents the cystoblast. T gives the total cells at the end of each division, and D gives the cystocyte division cycle. Single cells in the left column produce additional cells, if they divide completely; and two cell clusters, if they form ring canals. When the cells in a cluster divide, the new daughter cells either remain attached by ring canals or separate from the cluster as additional single cells. In (A) the single cells produced from a cluster are added to the single cells which have never been part of a cluster in the left column. All single cells are assumed to have an equal probability of forming a two-cell cluster during subsequent divisions. In (B) the single cells split off from a cluster form a separate pool of cells in the

of division. However, unlike wild type cystocytes, the cells generated will be found to be either unconnected or connected in abnormal small clusters (see Fig. 6 III).

The complete cytokinesis hypothesis provides two sources of single cells: they may be the direct descendants of the original cystoblast, or they may be budded off from the cystocytes of a cluster. Since these two groups of single cells may or may not have the same potential ability to start new clusters, there are two variations of this hypothesis.

In the first variation, all single cells, whether they are direct descendants of the cystoblast or of cystocytes in a cluster, have the same probability of forming a ring canal at the next mitosis. The exchange of cells between clusters and the pool of single cells is shown in Figure 7A.

In the second variation of the complete cytokinesis hypothesis, the cystoblast, and its direct descendants, which have never been part of a cluster, are the only single cells with the capacity of forming canals. A second pool of single cells is formed by the cystocytes that have been split off of clusters. These cystocystes retain the ability to divide, but not the ability to form ring canals. The predictions as to the relative numbers of both types of single cells and of cells in clusters are shown in Figure 7B. In this variation the percentage of cells belonging to the pool of single cells unable to form clusters increases with each cycle of division.

The relative frequency of clusters containing differing numbers of cystocytes in the germarium can be predicted using the binomial expansion. This process is repeated for each size cluster, and for each cycle of division (see Fig. 8). Clusters with the same number of cells may have different internal patterns, and

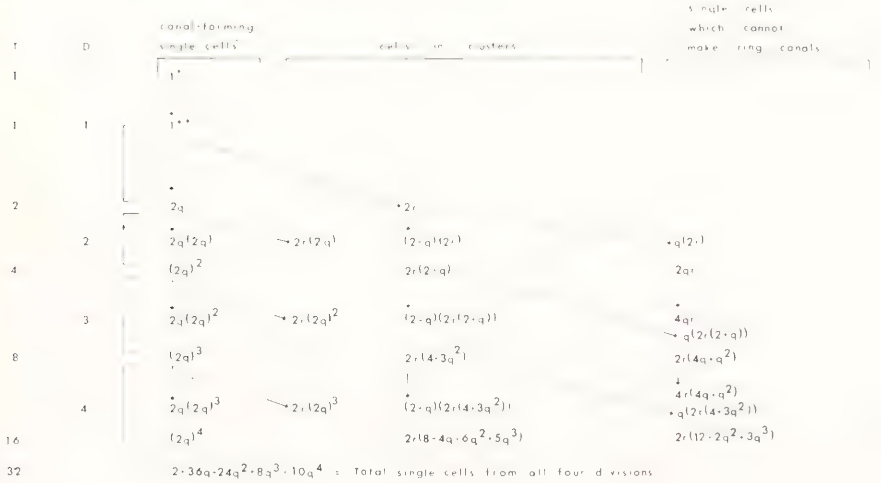
T
D
canal-forming single cells
cells in clusters
single cells which cannot make ring canals
1
1*
1
1
1**
2
2q
2r
2
2q(2q)
2r(2q)
(2-q)(2r)
q(2r)
4
(2q)²
2r(2-q)
2qr
3
2q(2q)²
2r(2q)²
(2-q)(2r(2-q))
4qr
q(2r(2-q))
8
(2q)³
2r(4-3q²)
2r(4q-q²)
4
2q(2q)³
2r(2q)³
(2-q)(2r(4-3q²))
4r(4q-q²)
q(2r(4-3q²))
16
(2q)⁴
2r(8-4q-6q²-5q³)
2r(12-2q²-3q³)
32
2-36q-24q²-8q³-10q⁴ = Total single cells from all four divisions

FIGURE 7B

right column. These single cells are assumed capable of further division, but incapable of forming ring canals in future divisions. In both diagrams the probable number of cells from all sources at the end of a division cycle is summed in each category. Arrows run from this sum to the probable distribution of these cells after a further cycle of division.

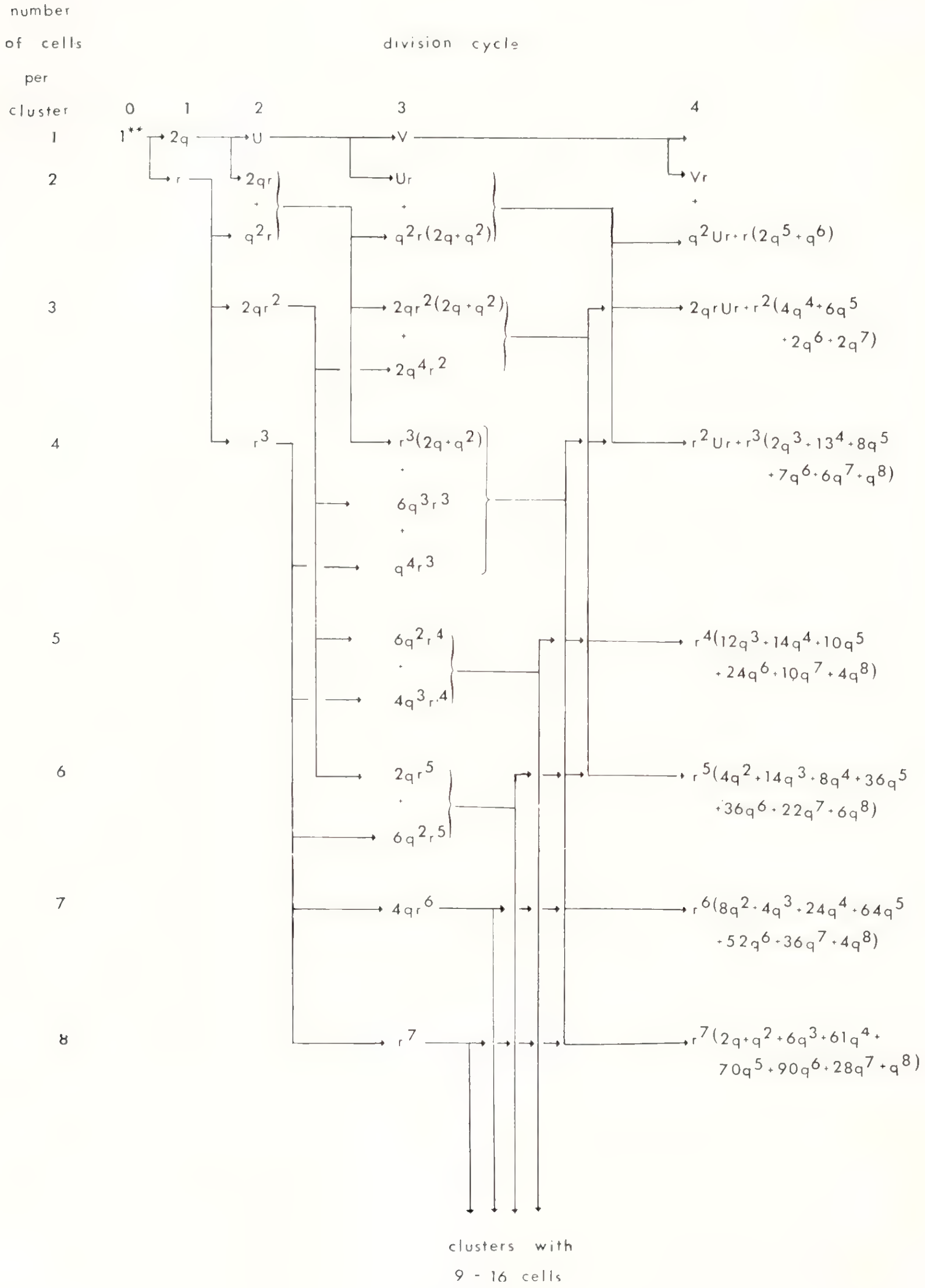
number of cells per cluster
division cycle
0
1
2
3
4
1
2
3
4
5
6
7
8
1^{**}
$2q$
r
U
$2qr$
+
q^2r
$2qr^2$
r^3
V
Ur
+
$q^2r(2q+q^2)$
$2qr^2(2q+q^2)$
+
$2q^4r^2$
$r^3(2q+q^2)$
+
$6q^3r^3$
+
q^4r^3
$6q^2r^4$
+
$4q^3r^4$
$2qr^5$
+
$6q^2r^5$
$4qr^6$
r^7
Vr
+
$q^2Ur+r(2q^5+q^6)$
$2qrUr+r^2(4q^4+6q^5+2q^6+2q^7)$
$r^2Ur+r^3(2q^3+13^4+8q^5+7q^6+6q^7+q^8)$
$r^4(12q^3+14q^4+10q^5+24q^6+10q^7+4q^8)$
$r^5(4q^2+14q^3+8q^4+36q^5+36q^6+22q^7+6q^8)$
$r^6(8q^2+4q^3+24q^4+64q^5+52q^6+36q^7+4q^8)$
$r^7(2q+q^2+6q^3+61q^4+70q^5+90q^6+28q^7+q^8)$
clusters with 9 - 16 cells

FIGURE 8.

may be formed during different cycles of division. We assume that exactly one additional cycle of division occurred in the *fes* germarium. Since the number of metaphases in a cluster indicates the number of cells formed in the previous division, the additional cycle of division in *fes* shows the configurations which were produced during the fourth cystocyte division. The predicted relative frequency for each size cluster was based on the sum of the predictions for all cycles of division, assuming the four cycles to be equally represented in the germarium. A test was calculated to determine which variation of the model gave the best fit to the overall relative frequencies of clusters of different sizes observed in the germarium.

Since single cells were the largest group observed, a value for q, the probability of complete cytokinesis, was calculated using the observed ratio of single cells to total cells. Single cells make up 38.4% of the total at 18° C, and 41.8% of the total cells at 25° C. Assuming equal representation of the four division cycles, there should be $2 + 2q(8 + 4q + 2q^2 + q^3)$ single cells for every 32 cells according to the first variation, and $2 + 36q - 24q^2 + 8q^3 + 10q^4$ single cells for every 32 cells according to the second variation of the complete cytokinesis hypothesis (see Fig. 7). Values of q were approximated by setting the observed ratio equal to the predicted ratio of single cells to cells in clusters and running a trial series of calculations to estimate q to three significant digits. Four values for q were approximated, one for each variation for the data at 18° C and at 25° C. These values of q were used to predict the number of clusters with two to eight cells in *fes* germaria from the two temperature series. There were too few observations of large clusters to warrant continuing the calculations.

In Table II the four sets of predictions are compared with the data collected from the colchicine-treated *fes* germaria. A chi-square value was calculated from the deviations between each set of predictions and the appropriate set of data. The number of clusters predicted by the first variation of this hypothesis was very highly significantly different from the observed data. The ratio of single cells to

FIGURE 8. Expressions for the frequencies of clusters with two to eight cells, after one to four cycles of division, starting from a single cystoblast (1**). The first row shows the frequencies of canal-forming single cells, and corresponds to the first column of Figure 7A or 7B. This frequency differs after the second division cycle according to which model is considered, and is represented by U or V after two or three divisions, respectively. U is $2q + 2q^2$ in the first model (Figure 7A) and $(2q)^2$ in the second model (Figure 7B). V is $4q + 2q^2 + 2q^3$ in the first model, and $(2q)^3$ in the second. The probability of complete cytokinesis is q, and $r = 1 - q$ is the probability that a cell will form a ring canal. Branching arrows run from the frequency of each given cluster to its corresponding frequency contributions in clusters of equal or greater sizes in the following division cycle. For example, after the second division cycle there are on the average $2qr + q^2r = r(2q + q^2)$ two cell clusters, and these lead to clusters of two, three or four cells at the third cycle in proportion of q^2, $2qr$ and r^2, respectively. Hence arrows lead from $2qr + q^2r$ (two-cell clusters at division cycle 2) to the proportionate terms $q^2r(2q + q^2)$, $2qr^2(2q + q^2)$ and $r^3(2q + q^2)$ at the third cycle. It should be kept in mind that the frequencies shown here are for clusters, rather than for cells in clusters as in Figure 7. Thus to obtain the frequency of cells in clusters of a given size it is necessary to multiply the cluster frequency by the number of cells in the cluster. For example, the total number of cells after two division cycles is found by summing $1(U) + 2(2qr + q^2r) + 3(2qr^2) + 4(r^3)$. The total is 4 under the first model and $4 - 2qr$ under the second, as it should be after two cell divisions. The difference is just the number of single cells that cannot form ring canals under the second model. These are not shown in this diagram.

cells in small clusters was too low with this set of calculations. The second variation of the hypothesis predicted values that were not significantly different from the observed data. Therefore, assuming equal representation of all four division cycles, the hypothesis that (1) some of the *fes* cystoblasts and cystocytes undergo complete cytokinesis and (2) that cells, which are split off cytocyte clusters, can only undergo complete cytokinesis is in best agreement with the data.

In the older literature many accounts have been published describing in various insects situations where sister germ cells are joined by canals (see review in King and Akai, 1971). A canal system similar to that connecting *Drosophila* cystocytes is found between sister spermatocytes in *Bombyx mori*. King and Akai have observed midbodies and contractile rings in partly cleaved sister cells (1971, their Fig. 3), and they suggest that material from the midbodies somehow crosslinks the component fibrils of the contractile rings and prevents them from closing down further. Subsequently the midbody dissolves, and the "stabilized" contractile ring serves as the canal rim. We propose that the contractile ring also serves as the organelle about which the ring canal is elaborated in *Drosophila melanogaster* and that the product of the *fes* gene functions in the stabilization of the contractile ring. The values calculated for the frequency of complete cytokinesis for *fes* cystocytes were 0.356 for females reared at 18° C and 0.403 for females reared at 25° C.

TABLE II

Comparison of the number of groups of one to eight cells observed in colchicine-treated fes *germaria, with the number of groups predicted in each category by both variations of the "complete cytokinesis" hypothesis. The probability that a* fes *cell will undergo complete cytokinesis is given by* q

	Cells per group	Groups observed	Groups predicted 1.	Groups predicted 2.
18° C	1	768	768.4	768.5
	2	127	225.0	120.0
	3	45	66.8	46.8
	4	40	48.2	50.0
	5	25	20.5	19.6
	6	13	15.6	21.0
	7	23	9.5	15.6
	8	11	5.6	10.6
			$q = 0.488$	$q = 0.356$
			$\chi^2 = 77.34$	$\chi^2 = 10.66$
			$p < 0.0001$	$p = 0.15$
25° C	1	588	587.6	589.0
	2	92	162.2	89.8
	3	37	48.4	36.7
	4	37	31.3	33.9
	5	21	13.5	14.6
	6	7	9.4	13.8
	7	12	5.4	9.5
	8	4	3.0	6.1
			$q = 0.527$	$q = 0.403$
			$\chi^2 = 47.67$	$\chi^2 = 8.37$
			$p < 0.0001$	$p = 0.3$

At both temperatures wild type flies give zero values. We conclude that the product of the mutant gene is unstable, particularly at higher temperatures, and that it is successful in preventing the complete closure of the contractile ring only a fraction of the time. The "immature" appearance of each canal rim observed in the reconstructured germarium (Fig. 3) is in harmony with the above hypothesis.

In the germinal syncytia observed in *Drosophila melanogaster* and other species, all of the cells sharing a common cytoplasm divide in synchrony. Since the division of all such cells apparently is controlled as a unit, it is reasonable to suggest that a cue which causes one or more of the interconnected cells to differentiate will generate a sequence of reactions that terminates the mitotic activity of all other cells in the cluster. If the formation of two cells with four ring canals during the fourth cystocyte division is the cue for the differentiation of the pro-oocytes, their differentiation may terminate further mitosis among the other 14 sister cells. In *fes* germaria cystocyte divisions would continue indefinitely, since cells with four canals are generated so infrequently.

However, in *fes* ovarioles we do find clusters in which cystocytes have differentiated into nurse cells (which presumably are incapable of mitosis) even though the cluster lacks pro-oocytes. The cue for nurse cell differentiation may be related to the volume of the cells in question. In the normal germarium cystocytes do not double their volumes between divisions, and consequently the volume of each individual cell is reduced with each division. The average cell in a sixteen cell cluster is only one-fifth the volume of the original cystoblast. In *fes* the frequency of clusters of nurse cells in the vitellarium is about ten times greater for flies reared at 18° C than at 25° C (King, Koch, and Cassens, 1961). We know that in the germarium mitoses occur with equal frequencies at both temperatures (Table I). Perhaps lowering the temperature slows down the growth between divisions, so that after a few divisions a critical minimum volume is reached. This then serves as a cue which causes the cell to enter the nurse cell developmental pathway.

The authors are grateful for the useful criticisms of an early draft of this paper provided by Drs. L. T. Douglas, E. A. Koch, P. A. Smith, E. B. Spiess, N. E. Welker, and J. M. Whitten. Mr. David Calhoun checked the calculations and made useful suggestions as to the textual exposition of the mathematics. The illustrations were inked by Mr. E. J. Pfiffner. This research was supported by Public Service Grant 5TL GM903 and National Science Foundation Grant GB29279.

SUMMARY

Females of *Drosophila melanogaster* homozygous for the autosomal, recessive gene *fes* are sterile, and their ovaries contain "tumorous" cysts that continue to grow mitotically and may eventually posses thousands of undifferentiated cells. To study the earliest steps in the formation of a *fes* "tumor" we determined the three dimensional interrelations of the cells in a single mutant germarium utilizing electron micrographs taken of serial ultrathin sections. This germarium contained a large number of unconnected cells and clusters made up of only a few interconnected cystocytes. The distributions of dividing cells in *fes* and wild type

germaria, some of which were treated with colchicine, were also studied. All of the cystocyte divisions take place in the anterior third of the wild type germarium. Here a few isolated metaphases were seen in stem line oogonia and cystoblasts, and the rest of the metaphase figures were found in groups of 2, 4, and 8 and presumably represented dividing cystocytes. Metaphases were found throughout the *fes* germarium. The number of isolated metaphase figures observed in mutant germaria was 15–20 times higher than in wild type. Metaphases were also found in groups. Clusters of two were twice as abundant in *fes* as in wild type, and clusters of four were equally abundant. Clusters of eight were seen about six times more often in wild type than in *fes,* but clusters of 3, 5, 6, 7, 9, 10, and 11 metaphases (which were never observed in wild type germaria) were found in *fes.* We estimated that the average *fes* cystocyte undergoes one supernumerary division before leaving the germarium.

We concluded that while all cystocytes undergo incomplete division in wild type germaria, a significant fraction of *fes* cystocytes undergo complete cytokinesis. An algebraic model developed from this hypothesis predicts the relative frequencies of single cells and clusters containing between 2 and 8 cells and enables us to calculate q, the probability that cystocytes will undergo complete cytokinesis. The predicted frequencies were not significantly different from those observed, and the hypothesis was also consistent with the observed rate of division found in the colchicine-treated ovaries and the patterns of cytocyte interconnections found in the reconstructed *fes* germarium. Germaria from *fes* females reared at 18° and 25° C gave q values of 0.356 and 0.403, respectively. The mitotic rates were the same at both temperatures. We conclude that the product of the *fes* + gene is required for the formation of a stable canal system and suggest that the product of the mutant gene is defective in this regard and thermolabile. The *fes* + substance may function to prevent the constriction of the contractile ring during cystocyte cytokinesis.

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AGGREGATION OF HORSESHOE CRAB (*LIMULUS POLYPHEMUS*) AMEBOCYTES AND REVERSIBLE INHIBITION OF AGGREGATION BY EDTA[1]

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Amebocytes comprise over 99 per cent of the cells in the hemolymph (blood) of the horseshoe crab, *Limulus polyphemus* (Fahrenbach, 1970). In its circulating phase, the amebocyte is a large, oval shaped, nucleated cell with characteristic cytoplasmic granules that obscure the nucleus. When removed from the circulation amebocytes rapidly aggregate and undergo vast morphological alterations including swelling, loss of granules, vacuolization, formation of filamentous pseudopods, and contraction (Levin and Bang, 1964b; Dumont, Anderson and Winner, 1966).

Functional parallels can be drawn between *Limulus* amebocytes (Levin and Bang, 1966) and mammalian platelets, and non-mammalian thrombocytes (Belamarich, Shepro, Fusari and Kien, 1966), all hemostatic cells specialized to aggregate in response to injury (Loeb, 1927). Unlike vertebrate coagulation that involves cell aggregation in addition to the formation of fibrin, the stemming of blood flow from injured *Limulus* vessels is solely due to the aggregation and contraction of amebocytes (Howell, 1885; Loeb, 1903–04, 1927; Maluf, 1939).

The biochemical aspects of amebocyte aggregation are not well understood. An endotoxin clottable protein originating from amebocytes was identified and its properties are being investigated (Levin and Bang, 1964a, 1968; Solum, 1970; Young, Levin and Prendergast, 1971), but the relationship of endotoxin clottable material to the hemostatic mechanism of *Limulus* has not yet been elucidated. Past studies of amebocyte aggregation employed methods that are essentially nonquantitative and utilized reagents that shed little light on the mechanism involved (Copley, 1947; Morrison and Rothman, 1957). With the exception of the possible participation of sulfhydryl groups (Bryan, Robinson, Gilbert and Langdell, 1964) little information is available on the components involved in the mechanism of amebocyte aggregation.

In the current work an attempt is made to study the mechanism of amebocyte aggregation utilizing the turbidimetric method originally developed by Born (1962) and O'Brien (1962) to follow mammalian blood platelet aggregation. This method, when adapted for the study of amebocyte aggregation, provides a quantitative system that permits reproducible measurements. The present study reports on the retardation of *Limulus* amebocyte aggregation by ethylene diaminetetraacetate (EDTA) and the restoration of aggregation to EDTA treated amebocytes by certain preparations from *Limulus* hemolymph. Some of the properties of an aggregation promoting material in *Limulus* hemolymph are also discussed.

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[1] This research was supported in part by grants from the USPHS, HL 10002–5 and HL 05411–12.

MATERIALS AND METHODS

Animals

All animals used in these experiments were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts, and measured 6–11 inches across the carapace. Horseshoe crabs were kept throughout the winter in an 800 gallon artificial sea water aquarium maintained at 58° F, and were fed shelled mussels, *Mytilus edulis.*

Glassware

Glassware and needles that contacted the hemolymph were silicon coated with Siliclad (Clay Adams).

Reagents

Physiological saline. A Tris-buffered saline solution was modified from Bryan, Robinson, Gilbert, and Langdell (1964) by mixing 0.51 M NaCl in a 9:1 ratio with 0.05 M Tris-HCl buffer at pH 7.8. The Tris-HCl buffer to saline ratio was established as the minimum concentration of Tris-HCl that would maintain pH 7.8 throughout amebocyte aggregation and retraction. The final concentration of NaCl in this mixture was 0.49 M, and whenever possible all solutions were made up in physiological saline. Imidazole-buffered saline, artificial sea water, and Tris-buffered *Homarus americanus* Ringer's solutions (Welsh and Smith, 1960) were also tested as diluents for aggregating amebocytes but Tris-buffered saline was found to be superior.

Buffered ethylene diaminetetraacetate (EDTA). Disodium EDTA was adjusted to pH 7.8 with 0.5 M NaOH and then diluted to a final concentration of 0.1 M with physiological saline. Buffered sodium citrate and oxalate solutions were prepared by a similar procedure.

Apparatus and assay for aggregation

A Chrono-log Platelet Aggregometer was adapted for the study of amebocyte aggregation by addition of an overhead rotary motor equipped with a polyethylene paddle, and a reverse thermocouple to regulate the temperature of the curvette. Aggregation was measured as changes in turbidity at 610 nm and recorded as per cent transmission by means of an attached recorder usually run at 1 inch per minute. Saline (2.0 ml) was added to the cuvette and equilibrated to 15° C while being stirred at 300–335 rpm. Material to be tested for reversal of aggregation inhibition was diluted in saline and treated the same way. The recorder was run for 1 minute to establish a baseline transmission before the addition of amebocytes.

Hemolymph was routinely withdrawn from the articular sinus of the legs with a siliconized, 1 inch, 18 gauge needle. To observe uninhibited aggregation, 4 ml of physiological saline at 15° C were used as diluent for 1 ml of hemolymph, whereas in inhibition studies, 4 ml of "inhibitor" were previously drawn into the syringe. After the hemolymph—saline or hemolymph—inhibitor was mixed by inversion of the syringe, 1 ml of the mixture was discarded and approximately

0.3 to 0.8 ml was added directly to the cuvette. Less than 10 seconds elapsed between puncture of the articular sinus and the addition of amebocytes to the cuvette.

Because the concentration of amebocytes was not constant in samples of a given volume, a range of 0.3 to 0.8 ml of hemolymph was added from the syringe to the cuvette. This was done to make the final concentrations of amebocytes in the cuvette as nearly equal as possible. However, the final concentrations of inhibitor could only be calculated approximately. For example, 10^{-1} M EDTA was diluted to 80 mM with hemolymph, but after the additional dilution in the cuvette, the final concentration of EDTA was approximately 17 ± 6 mM. Similarly, the agents tested for reversal of aggregation inhibition were diluted again by 0.3 to 0.8 ml of hemolymph in inhibitor. Unless stated otherwise, the range of concentrations indicates final, but approximate, concentrations.

Proportionality between O.D. at 610 nm and the concentration of unaggregated cells forms the basis of the turbidimetric method of measuring cell aggregation (Born and Cross, 1963). A standard curve relating the concentration of unaggregated amebocytes to O.D. 610 nm was prepared by counting the number of amebocytes treated with 10^{-1} M N-ethylmaleimide (NEM) at specific O.D.'s. In a given experiment, ameboycte aggregation in saline at 15° C was considered a control, the maximum aggregation for that particular animal. Quantitative comparison of aggregation data from different experimental samples or different animals is achieved by either of two methods: (1) Aggregation is expressed directly as changes in per cent transmission when the number of amebocytes in the samples is approximately equivalent. (2) In samples with different amebocyte concentrations, aggregation, recorded as per cent transmission, is converted to O.D. at 15 sec intervals and then expressed as a percentage of the control:

$$\frac{\Delta\ \text{O.D.}_{\text{at time t 100 ambeocytes (experimental)}}}{\Delta\ \text{O.D.}_{\text{at time t/100 amebocytes (control)}}} \times 100 = \Delta\ \%_{\text{O.D.}}$$

Preparations

Serum. Serum was prepared from hemolymph (10 ml) withdrawn by cardiac puncture the prosoma-opisthosma junction with an 18 gauge, 1″ needle. The hemolymph was transferred from the syringe to 15 ml polytheylene tubes at room temperature. When retraction of the amebocyte aggregate was fairly complete (15 min), the cellular mass was removed and the resulting serum centrifuged at 1760 × g for 5 min at 4° C to remove any residual cellular material. Serum is stable for 1 week or more at 4° C, and active for periods of 6 months or longer when stored frozen.

Cell free plasma. Cell free plasma was prepared by a procedure modified from Levin and Bang (1964a). Hemolymph (10 ml) was collected into an ice-cold syringe by cardiac puncture from a *Limulus* that was precooled at 4° C for 24 hours. Amebocytes were removed by centrifugation at 0° C in ice-cold centrifuge tubes until the centrifugal force equalled 12,100 × g. Approximately 8 ml of the resulting plasma supernatant was drawn off and stored either at 4° C or frozen.

Amebocyte homogenate supernatant (AHS). The amebocyte pellet from the preparation of cell free plasma was first washed twice, without resuspension, with

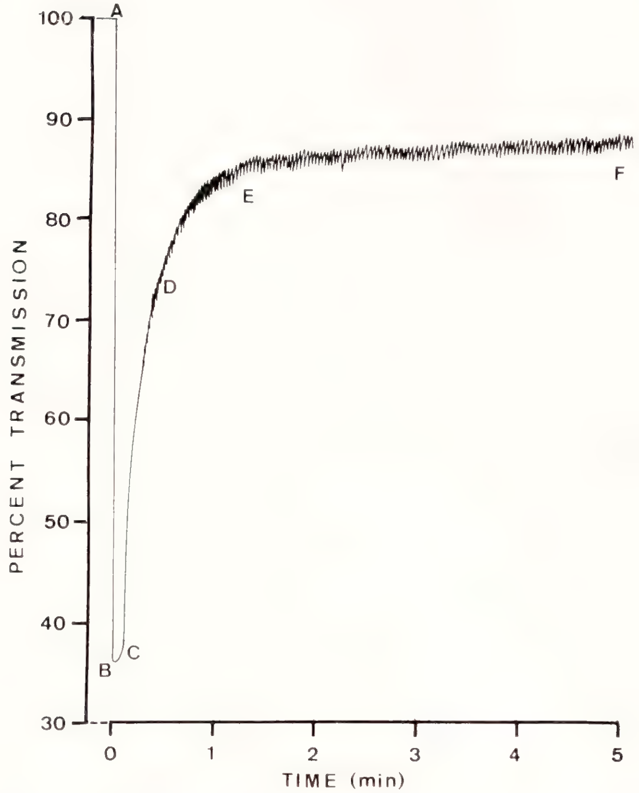


FIGURE 1. Typical photometric record of amebocyte aggregation at standard conditions. Physiological saline at 15° C is the diluent and stirring speed is 300–335 rpm. Records of aggregation show reproducible characteristics; decrease in transmission resulting from addition of amebocytes to the cuvette (A–B); a lag period (B–C); rapid rise in transmission (C–D); levelling off period (D–E); a plateau period (E–F). When transmission is converted to O.D. and aggregation is expressed as per cent change in O.D., the resulting curve has the same shape as that shown above.

1.0 ml cold saline and then frozen in 1.0 ml saline. After thawing, the pellet was homogenized by rotary motor at 1000 rpm or by hand for 5 min employing a ground glass homogenizer and teflon pestle. Freeze-thawing alternated with homogenization was repeated twice more. During homogenization, the homogenate was kept on ice and precautions taken to prevent foaming. The final volume of the homogenate was 2 ml, which represents approximately a 5-fold concentration of amebocytes in the original volume of hemolymph. The amebocyte homogenate was then centrifuged at 0° C at 12,100 × *g* for ten minutes. After centrifugation, the slightly opaque supernatant was drawn off and examined under a phase microscope to make certain that cellular fragments were absent.

The AHS yield was 1.5–1.8 ml for each 2 ml of homogenate and 0.5 ml aliquots were stored at –20° C.

RESULTS

Characteristics of amebocyte aggregation in saline

When measured photometrically, amebocyte aggregation exhibits the characteristics shown in the typical aggregation graph in Figure 1. The sharp decrease in transmission that results from addition of amebocytes to the cuvette is followed by a short lag period, lasting fewer than 15 seconds, in which only a 0–2% rise in transmission occurs. Transmission rapidly rises following this lag period and 80–100 per cent of the total change in transmission takes place in the first 30 seconds. Over the next 30 sec, increases in transmission level off, and during the last 4 min, a plateau occurs where only slight changes in transmission are observed. Amebocyte aggregation can therefore be considered fairly complete at 1 min after

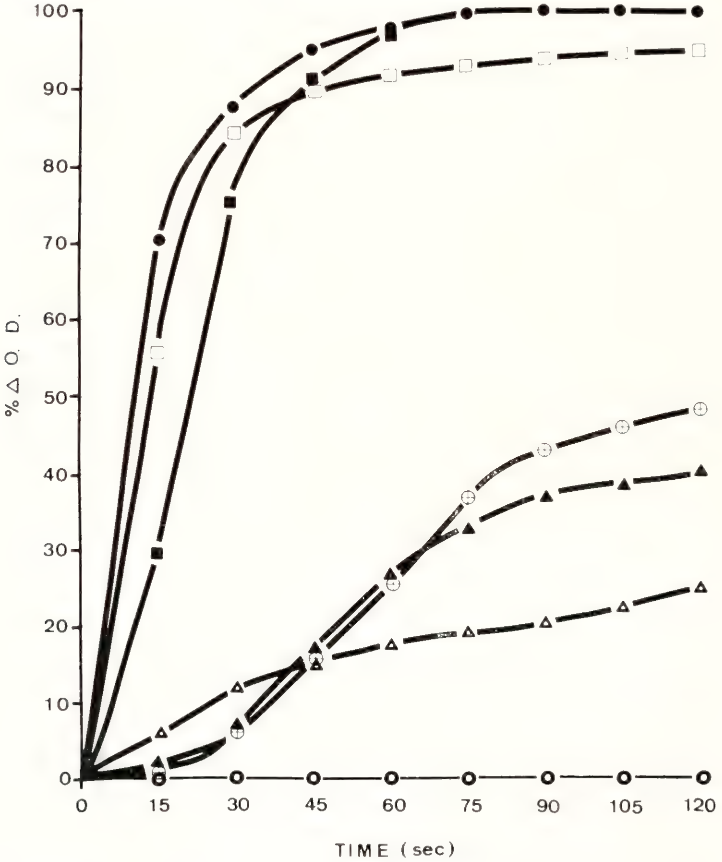


FIGURE 2. Typical inhibition of amebocyte aggregation by EDTA and citrate. Control aggregation in saline (●); amebocytes in: EDTA, 17 ± 6 mM (○); citrate, 17 ± 6 mM (△); EDTA, 8 ± 3 mM (▲); citrate, 8 ± 3 mM (⊕); EDTA, 2 ± 1 mM (□); citrate, 2 ± 1 mM (■). Concentrations of EDTA and citrate are approximate, but final concentrations in the cuvette.

the addition of amebocytes to the cuvette. During the plateau period, the recorder pen often oscillates with excursions of varying amplitude, presumably produced by free aggregates passing through the light path.

It was determined that the reproducibility of aggregation measurements is critically dependent on the temperature and the speed of stirring. Optimal aggregation occurs at 15° C and this temperature is routinely employed for measurement of amebocyte aggregation. Lower temperatures (10° C, 5° C, and 0° C) produce progressive inhibition of both the rate and extent of aggregation measured over 2 minutes. Microscopic examination of amebocytes at 0° C shows the presence of aggregates ranging in size from approximately 2–50 cells. Amebocytes within these aggregates retain their individual identity and pseudopods are only occasionally seen at the periphery of the aggregates. Stirring speed has a biphasic effect on aggregation. Low speed stirring (60–300 rpm) enhances the rate of aggregation while higher stirring speeds (335–450 rpm) appear to inhibit aggregation. A stirring speed of 300–335 rpm is routinely employed. Variation in the number of amebocytes within that range suitable for photometric studies (700–1600 cells/mm^3) has no effect on the pattern of aggregation.

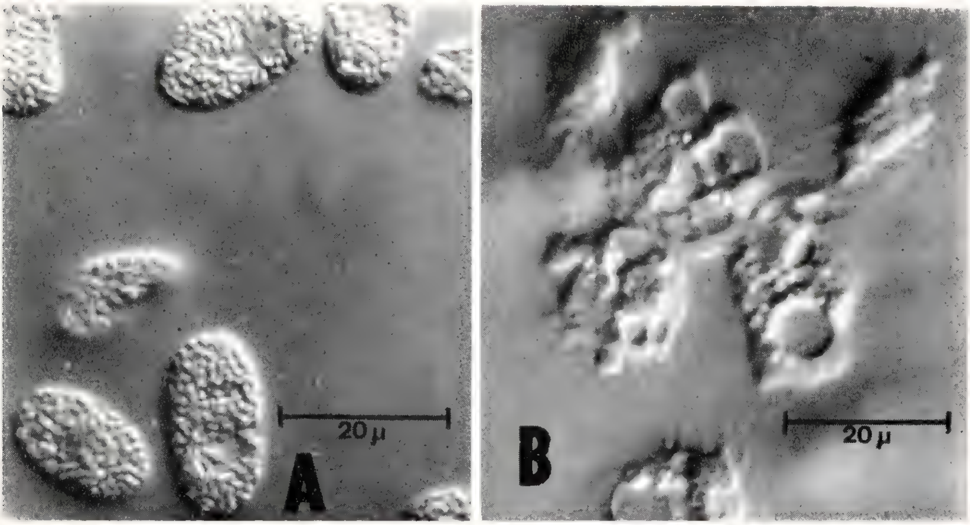


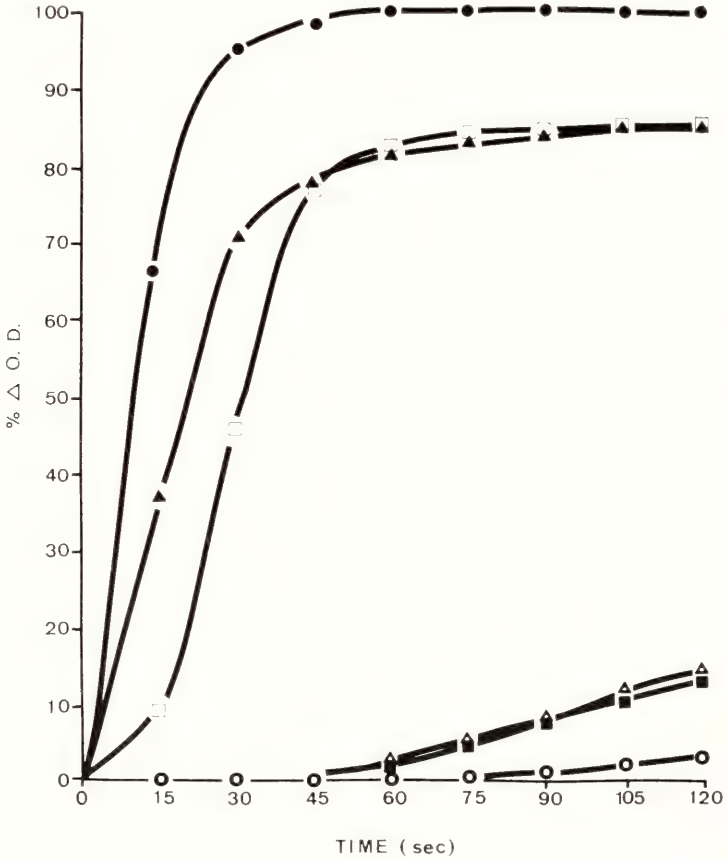
FIGURE 3. Photomicrographs of amebocytes 2 minutes after withdrawal into a, EDTA; b, saline (Nomarski Optics).

Inhibition of amebocyte aggregation by EDTA

Buffered EDTA at final concentrations of 17 ± 6 mM completely inhibits increases in transmission over the first two minutes (Fig. 2). In 8 ± 3 mM EDTA, inhibition of aggregation is significantly decreased and when the concentration of EDTA is reduced to 2 ± 1 mM, inhibition is negligible. Buffered sodium citrate at the same concentrations is less effective than EDTA, but inhibition of aggregation produced by both citrate and EDTA is concentration dependent (Fig. 2).

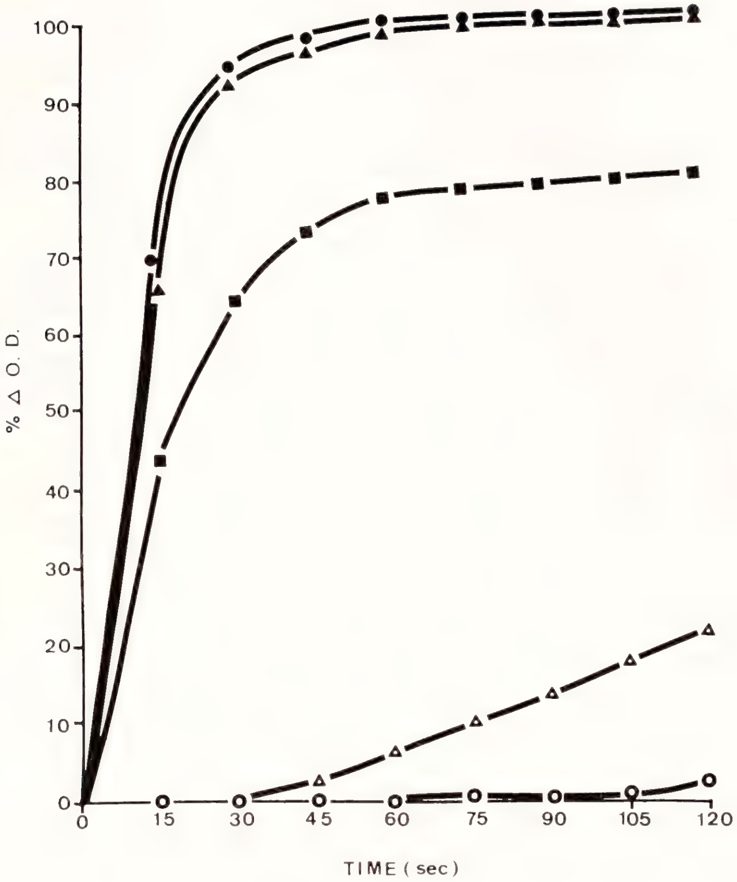
Oxalate inhibition of amebocyte aggregation is not suited to photometric examination, for, even at low concentrations (2 ± 1 mM), buffered oxalate causes expulsion of intact, free granules that produce turbidity and mask increases in transmission resulting from aggregation. As evaluated by microscopic observation, oxalate inhibition of amebocyte aggregation is intermediate between the inhibition produced by EDTA and citrate.

When examined microscopically amebocytes in 17 ± 6 mM EDTA appear intact and show little evidence of the extensive alterations characteristic of aggregation (Fig. 3). EDTA treated amebocytes remain ovoid and contain the characteristic granules (Fig. 3A), whereas, amebocytes in saline at 2 min (Fig. 3B)



4a.

FIGURE 4. Representative experiments showing the effect of calcium (4a) and magnesium (4b) on amebocytes in EDTA; (4a) control aggregation in saline (●), amebocytes in 17 + 6 mM EDTA (○); amebocytes in 17 + 6 mM EDTA added to: 8 ± 1 mM calcium (△); 15 ± 2 mM calcium (■), 24 ± 2 mM calcium (▲), 32 ± 3 mM calcium (□). (4b) Control aggregation in saline (●); amebocytes in 17 ± 6 mM EDTA (○). Amebocytes in 17 ± 6 mM EDTA added to: 8 ± 1 mM magnesium (△), 15 ± 2 mM magnesium (■), 24 ± 2 mM magnesium (▲).



4b.

FIGURE 4 (*continued*).

exemplify the typical morphological alterations of aggregation (Dumont, Anderson and Winner, 1966). Not infrequently, EDTA preparations have amebocytes that are more round than elliptical and a few free intact granules are present outside of the cells.

Although EDTA at 17 ± 6 mM markedly inhibits amebocytes aggregation, this inhibition is perhaps more aptly termed a retardation of aggregation because, with time, some aggregation takes place in EDTA. During the first 2 min after addition of amebocytes in EDTA to the cuvette, a slight rise (0–2%) in transmission frequently occurs, but over 5 min period increases in transmission are usually significant (0–15%). Furthermore, after stirring on the aggregometer for 5 min, microscopic examination of amebocytes in EDTA reveals aggregates even when no rise in transmission is observed. These aggregates range from small (approximately 2–20 cells) to medium sized (100 cells), and the amebocytes within these aggregates retain their identity with pseudopods evident only on those cells at the periphery of the aggregates. Lower concentrations on EDTA (in the range of 14 ± 5 mM) are often as effective as 17 ± 6 mM EDTA in inhibiting amebocyte aggregation for 5 minutes but EDTA at final concentrations of 17 ± 6 mM is employed routinely.

The transistory nature of EDTA inhibition eliminates the possibility of employing standardized cell pools of known amebocyte number in aggregation experiments, for even if EDTA preparations are kept on ice with very gentle agitation to prevent sedimentation, most of the amebocytes are in small to medium sized aggregates within 15–20 minutes.

Effect of calcium and magnesium of amebocytes in EDTA

Calcium and magnesium produce aggregation of amebocytes in EDTA (Fig. 4). Minimum final concentrations of 8 ± 1 mM calcium or magnesium cause only slight

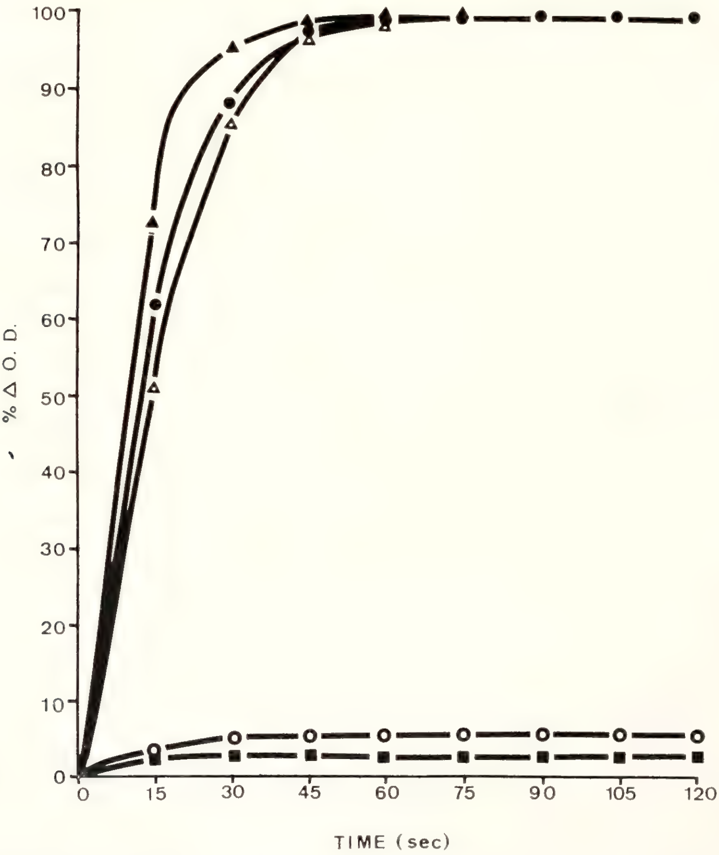


FIGURE 5. Effect of serum, AHS and cell free plasma on amebocytes in EDTA. Data from experiments on 3 different animals. Control aggregation in saline (●). Amebocytes in 17 ± 6 mM EDTA (○); amebocytes in EDTA with 1:20 diluted AHS (△); amebocytes in EDTA wtih 1:20 diluted serum (▲); amebocytes in EDTA with 1:20 diluted cell free plasma (■).

aggregation of amebocytes in EDTA but when magnesium levels are raised to 24 ± 2 mM, aggregation is complete within 30 sec. At similar concentrations, magnesium is more effective than calcium in reversing EDTA inhibition; calcium at 32 ± 3 mM produces only partial aggregation of amebocytes in EDTA. Potassium chloride in final concentrations of up to 32 ± 3 mM does not cause aggregation of EDTA inhibited amebocytes. Based on the results of 7 experiments that are similar to those shown in Figure 4, at least equivalent, if not excess magnesium is apparently required to reverse EDTA inhibition of amebocyte aggregation.

Reversal of EDTA inhibition

Addition of amebocytes in EDTA to a 1:20 dilution of either serum or Amebocyte Homogenate Supernatant (AHS) results in immediate aggregation of ameboyctes in EDTA (Fig. 5). The fall in optical density accompanying aggregation of amebocytes in EDTA by AHS or serum is indistinguishable in rate and extent from control aggregation in saline. Comparable dilutions of cell free plasma do not produce significant aggregation of EDTA inhibited cells.

Reversal of EDTA inhibited amebocyte aggregation by serum and AHS is not dependent either on the actual removal of EDTA, or on the effective removal of the chelating agent by addition of excess calcium or magnesium. The data in Figure 6 shows that if amebocytes withdrawn in EDTA are separated by gentle centrifugation at 4° for 30 sec at 280 × g, and then resuspended in fresh cell free plasma at 15° C, slow but spontaneous, aggregation takes place. When these resuspended, EDTA treated amebocytes in plasma are added to a 1:20 dilution of serum, aggregation is identical to control aggregation in saline. However, addition of amebocytes in EDTA to a 1:20 dilution of AHS or serum (not shown in Fig. 6) also produces aggregation that is indistinguishable from control aggregation.

The effect of temperature on serum induced aggregation of amebocytes in EDTA is similar to the effect of temperature on aggregation in saline; the temperature optimum for both is 15° C, and with lower temperature there is a concommitant decrease in the rate and extent of aggregation. At 0° C, serum—EDTA amebocyte samples consistently show a considerable lengthening of the lag period to 1 min or longer. Microscopic examination of these samples reveals small aggregates of amebocytes that retain their identity.

Some properties of serum and AHS

The potency of serum from different animals varies. Good reversal of EDTA inhibition is observed with dilutions of serum as great at 1:400 but, in general, the activity of serum begins to decline when dilution with saline is greater than 100-fold. AHS can often be diluted as much as 800 times before any decrease in aggregating activity is observed.

Samples of serum and AHS frozen for as long as 8 months still retain excellent activity. After 5 days at 4° C, activity of serum is generally unchanged, but AHS activity is diminished and a white insoluble precipitate forms within 2–12 hours at 4° C or after refreezing. Activity of serum and AHS is reduced or lost completely within 3–10 hours at room temperature. After 5 minutes in boiling water, serum is completely inactive, but AHS at 1:10 dilution is observed to retain some very slight portion of activity. The aggregating activity of serum is unchanged after exposure to unsiliconized glass beads, and after prolonged dialysis at 4° C against physiological saline. Dialysis usually results in some reduction of the activity of AHS. Quantitative effects of dialysis on AHS are difficult to assess, however, because of the precipitate formed in the process of dialysis. The activity of serum or AHS is essentially non-dialyzable and heat labile. Therefore, the ability of these preparations to reverse EDTA inhibition of aggregation is not simply a result of the reversal of the effects of EDTA by free calcium and/or magnesium.

Aggregation promoting material: evidence for release and participation in amebocyte aggregation

Several experiments indicate the aggregation promoting activity of serum is produced by a substance (or substances) released from amebocytes during aggregation. If amebocyte aggregation is retarded by low temperatures, the supernatant (cell free plasma) does not cause aggregation of amebocytes in EDTA. Moreover, titration of the sera formed in aliquots of a hemolymph sample shows that as amebocytes aggregation proceeds with time in unagitated samples (up to 5 hours) there is increased aggregating activity in the serum.

Although exogenous cell free plasma has no effect on control aggregation in saline, the influence of plasma on the aggregating activity of AHS was examined. AHS is titrated to the lowest dilution that exhibits submaximal activity (1:800) and then incubated with varying concentrations of cell free plasma. No effect in the activity of the diluted AHS is noted after incubation with as much as 4 volumes cell free plasma.

The aggregation promoting material (APM) in serum and AHS may participate in amebocyte aggregation. When amebocytes in saline are added to a 1:20 diluted serum prepared from the hemolymph of another *Limulus*, aggregation is enhanced compared with control aggregation in saline alone. Withdrawal of hemolymph directly into dilute heterologous serum also enhances aggregation. The increase in aggregation by exogenous APM implies that endogenous APM may operate in the mechanism of amebocyte aggregation.

Mode of action of EDTA inhibition

To test for a direct effect of EDTA on the aggregation promoting material, amebocytes treated with EDTA are added to a 1:20 dilution of serum and increasing concentrations of EDTA (4 ± 1 mM to 71 ± 6 mM). Under these conditions, there is a progressive decrease in aggregation of EDTA cells that is correlated with increasing EDTA concentration. This decrease in restorative activity indicates that EDTA inhibits the aggregating activity of serum. But when serum, treated with an equal volume of 100 mM EDTA, is dialyzed against saline at 4° C, there is no effect on the aggregating activity of the serum. Therefore, if EDTA does directly inhibit the activity of the aggregation promoting material, such inhibition results in no permanent loss of activity.

To examine the possibility that EDTA could retard amebocyte aggregation by preventing release of the aggregation promoting material from amebocytes, several approaches were employed. First, an attempt was made to isolate the active material from amebocytes treated with EDTA. Hemolymph was withdrawn into 100 mM EDTA so that the final concentration with amebocytes was 75 mM. After separation of the amebocytes by centrifugation, the EDTA—plasma was drawn off and the amebocyte pellet was washed and AHS prepared. The supernatant of this homogenate produces reversal of EDTA inhibition, and despite an extended lag period, the aggregation is complete. From these results it appears that amebocytes in EDTA do contain the APM.

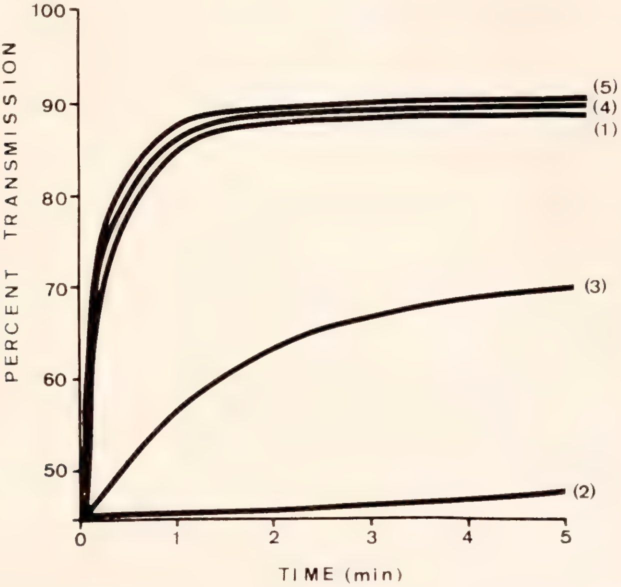


FIGURE 6. Tracings of representative graphs showing aggregation of EDTA inhibited amebocytes. Graphs are modified to smooth out oscillations; control aggregation in saline (1); amebocytes in 17 ± 6 mM EDTA (2); amebocytes withdrawn in EDTA and resuspended in cell free plasma (3); amebocytes withdrawn in EDTA, resuspended in cell free plasma, and added to 1:20 dilution of serum (4); amebocytes in EDTA added to 1:20 dilution of AHS (5).

A second approach employed to investigate possible inhibitory effects of EDTA on the release of APM from amebocytes involved direct assay of the supernatants of EDTA treated amebocytes. The ability of these supernatants to restore aggregation to amebocytes in EDTA is compared to that of the control supernatants of amebocytes treated with saline. Hemolymph is withdrawn in a 1:3 ratio with 100 mM EDTA, mixed and the suspension divided into equal aliquots in polyethylene centrifuge tubes. After the amebocytes are separated by centrifugation, the EDTA-hemocyanin supernatant is carefully removed, and the pellet of EDTA treated amebocytes covered with 0.2 ml cell free plasma and 0.8 ml physiological saline at room temperature. A total of 4 min elapsed between the time of puncture and the replacement of the supernatant by saline—plasma. The cell pellets are left in contact with the plasma—saline supernatants at room temperature for

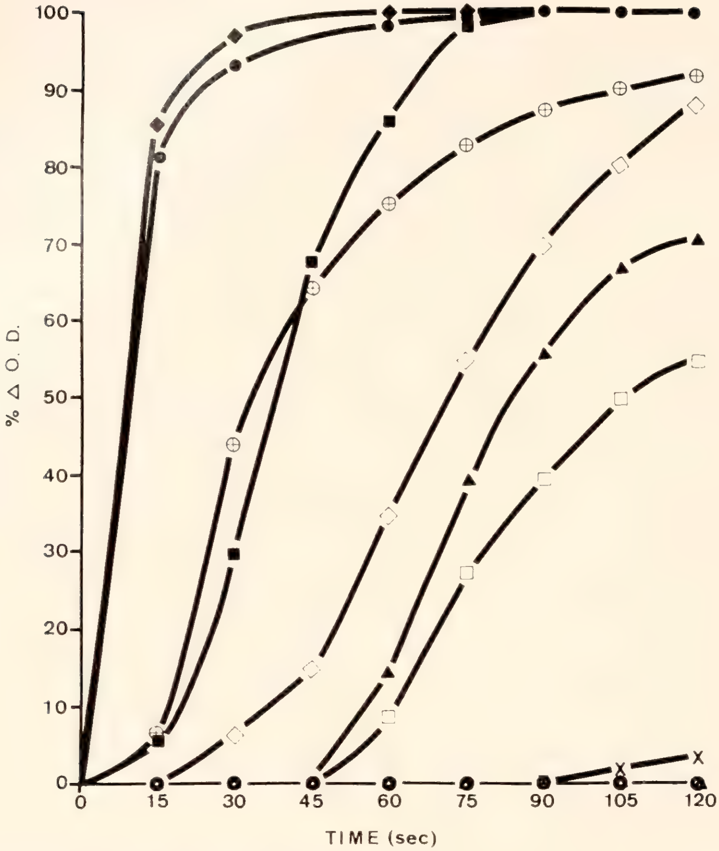


FIGURE 7. Typical experiment showing the effect of EDTA on the release of aggregation promoting material from amebocytes. Control amebocyte pellet represents amebocytes withdrawn in saline. EDTA amebocyte pellet represents amebocytes withdrawn in 75 mM final EDTA. At 0 time (4 min after puncture) supernatants of both pellets contained 0.2 ml inactive plasma + 0.8 ml saline, and did not produce significant aggregation of amebocytes in EDTA (×); aggregation in saline (control) (●); amebocytes in 17 ± 6 mM EDTA (○). Amebocytes in EDTA added to 1:20 diluted supernatants of: control amebocyte pellet at 1′ (▲); control amebocyte pellet at 10′ (■); control amebocyte pellet at 20′ (◆); EDTA amebocyte pellet at 1′ (△); EDTA amebocyte pellet at 10′ (□); EDTA amebocyte pellet at 20′ (◇); EDTA amebocyte pellet at 30′ (⊕).

periods of 1, 10, 20, and 30 min, at which times the supernatants are removed, checked microscopically to insure the absence of cells or cell fragments, and assayed for ability to restore aggregation to amebocytes in EDTA. At each time interval, the activity of the supernatants of EDTA treated pellets is compared with the control, saline—plasma supernatant of amebocytes originally withdrawn in a 1:3 ratio with saline. The results of a representative experiment (Fig. 7) show that at any given time the supernatant of the control amebocyte pellet is significantly more active in producing aggregation of amebocytes in EDTA than the supernatants of the EDTA treated amebocyte pellet. Reversing activity is present in the control supernatant at 1 min, and at 20 min, aggregation of amebocytes in EDTA induced by this supernatant is equivalent to control aggregation in saline. However, activity only becomes evident in the supernatant of the EDTA treated pellet at 10 min, and not until 30 min did this supernatant produce complete but slow aggregation of amebocytes in EDTA.

The results of this experiment suggest that exposure to EDTA retards the release of APM from amebocytes. It could be argued that residual EDTA in the amebocyte pellet causes direct inactivation of APM and produces this effect, but this experiment has been performed with the same results using once-washed, EDTA treated amebocytes. The pellet method is preferred for this type of experiment to minimize disruption of amebocytes caused by resuspension.

Discussion

Amebocyte aggregation followed photometrically shows the basic characteristics of platelet aggregation (Skoza, Zucker, Jerushalmy and Grant, 1967). As a measure of aggregation, increases in transmission are quantitative and highly reproducible, but in so far as the manner in which these changes in transmission depend on the size and number of platelet aggregates, the photometric method remains empirical. From a study of the relationship between aggregate size and number and the observed changes in transmission, Born and Hume (1967) concluded that the relationship between platelet aggregation and observed changes in transmission involved both the formation of aggregates and the increasing density (contraction) of the aggregates. In view of the similarity between photometrically measured platelet and amebocyte aggregation, contraction of large amebocyte aggregates may be responsible for the characteristic rapid rise in transmission. Combination of single amebocytes to form small aggregates and formation of larger aggregates from small amebocyte aggregates would take place in the lag phase where transmission changes are slight.

In agreement with previous reports (Loeb, 1927; Copley, 1947) it was demonstrated that low temperatures retard the aggregation of *Limulus* amebocytes. Microscopic examination of amebocytes in saline reveals, however, that some aggregation can still take place at 0° C. In light of the findings of Born and Hume (1967), it might be assumed that aggregate contraction that would produce rapid increases in transmission is more affected by low temperatures than is the cohesion of individual amebocytes.

As a system for the study of the mechanism of amebocyte aggregation, inhibition by EDTA is somewhat limited in that aggregation does eventually occur, but EDTA is superior to the only other known aggregation inhibitor, N-ethylmaleimide, as the latter produces irreversible inhibition of amebocyte aggregation (Byran, Robinson, Gilbert and Langdell, 1964; Levin and Bang, 1966). Nevertheless, EDTA treated amebocytes are morphologically intact, usually remaining as single cells for 2 min or longer when stirred, and aggregate in a manner indistinguishable from aggregation in saline when diluted preparations of serum or AHS are added.

It is likely that EDTA "halts" amebocyte aggregation in its early stages rather than prevents the initiation of aggregation. When amebocytes are withdrawn in saline and then added to EDTA (or citrate) at a final concentration of

79 ± 7 mM, some aggregation takes place in the first 15 seconds, but after this time aggregation is significantly retarded, (unreported observations). Indeed, Dumont, Anderson and Winner, (1966) clearly demonstrated that in samples of aggregating amebocytes all cells do not undergo aggregation simultaneously, and that as late as 30 minutes after withdrawal of the hemolymph there were a few amebocytes that still remained ovoid and possessed characteristic cystoplasmic granules.

It should be made clear that because there is no fibrous protein of plasma origin analogous to the vertebrate fibrinogen—fibrin conversion in the hemostatic mechanism of *Limulus* (Howell, 1885; Loeb, 1903, 1927), a serum by traditional definition, does not form (Maluf, 1939). However, since the amebocyte cellular mass ("pseudocoagulum") retracts, consolidating the coaglum and squeezing out the non-cellular hemolymph, the term serum is used to describe the hemocyanin containing fluid remaining after amebocytes have aggregated and retracted. The supernatant of aggregated, retracted amebocytes has also been termed "pre-gel" by Levin and Bang, (1964a), but this term appears to be limited.

An aggregation promoting material may be involved in the mechanism of amebocyte aggregation. Not only is the rate and extent of serum restored aggregation of EDTA treated amebocytes strikingly similar to aggregation in saline but the temperature optimum for both types of aggregation is 15° C. Moreover, lowered temperatures inhibit both serum-restored and control aggregation in saline. Morphological observations of amebocyte aggregation in either saline or EDTA-serum preparations at 0° C suggest that aggregate contraction is more effectively inhibited by low temperatures than is the cohesion of individual amebocytes to form aggregates. Enhancement of aggregation in saline by additional serum directly supports the involvement of endogenous aggregation promoting material in the mechanism of amebocyte aggregation. Furthermore, when the amebocytes of an occasional *Limulus* failed to aggregate in saline, addition of active serum prepared from the hemolymph of another *Limulus*, without exception, immediately restored aggregation to these deficient amebocytes.

Inhibition of amebocyte aggregation by EDTA (and citrate) implicates calcium and/or magnesium in the events leading to amebocyte aggregation. Divalent cations, particularly calcium, seem to be required in the mechanism of cell aggregation of some other invertebrate species which, like *Limulus*, rely on the aggregation of hemostatic cells to halt the flow of blood from injured tissues (Bookhout and Greenburg, 1940; Boolootian and Giese, 1959; Noble, 1970). Calcium is essential for mammalian blood platelet aggregation induced by ADP, and calcium binding compounds such as EDTA, citrate, oxalate, and EGTA (ethyleneglycol diaminoethyl tetraacetate) inhibit this reaction (Born and Cross, 1963; Hovig, 1964; Skoza, Zucker, Jerushalmy and Grant, 1967; Ardlie, Nishizawa and Guccione, 1970).

At equivalent concentrations, EDTA is more effective in retarding amebocyte aggregation than citrate but the retardation of aggregation produced by both citrate and EDTA is concentration dependent (Fig. 2). Substantial differences in the stability constants for calcium ($10^{-10.59}$ for EDTA compared to $10^{-3.22}$ for citrate; Des Prez, Bryant, Katz and Brittinghan, 1967) and the fact that EDTA chelates both calcium and magnesium could explain the increased effectiveness of EDTA in inhibiting aggregation. Upon addition to equvalent, if not excess magnesium, amebocytes in EDTA aggregate, whereas, only partial restortation of aggregation is produced by equimolar calcium (Fig. 4). Hovig (1964) observed a similar phenomenon in EDTA treated rabbit platelets and postulated that addition of magnesium to EDTA preparations could liberate substantial amounts of ionized calcium from Ca · EDTA complexes by virtue of the ionic equilibria involved.

However, if EDTA specifically and exclusively inhibits amebocyte aggregation by chelation of divalent cations, the restoration of aggregation to amebocytes in EDTA by aggregation promoting material in serum or AHS is difficult to explain. First, the aggregating activity of serum or AHS is essentially heat labile and nondialyzable. Furthermore, serum and AHS are fully active at dilutions 1:100 and 1:400, respectively. Such dilutions would lower calcium and magnesium in serum from the reported values of 10 and 46 mM (Robertson, 1970) to levels that are experimentally shown to be too low to cause any aggregation of amebocytes in EDTA (Fig. 4). Thus, restoration of aggregation to amebocytes in EDTA could not be produced solely by simple addition of divalent cations in serum and AHS. It appears then, that inhibition of amebocyte aggregation by EDTA may be the result of non-specific or generalized effects of EDTA on amebocytes, as well as the specific chelation of divalent cations (see Weiss, 1960).

Although information on non-specific effects of EDTA is sparse, it has been reported that EDTA can form metal chelates with trace, divalent ions and the rare earth metals (Johnson, 1955; according to Weiss, 1960). There is also evidence that EDTA produces effects on diverse biological systems that cannot be explained by simple chelation of ionized calcium, magnesium, or trace ions (Blithell, 1964; Levin and Bang, 1964a, Walters, 1969; Noble, 1970). Furthermore, Rossi (1967) demonstrated that EDTA treated human platelets exhibited increased consumption of glucose as well as an alteration in the synthesis of organic phosphates and suggested that a generalized effect of EDTA on some metabolic enzymes might account for this observation.

Whatever the mechanism of EDTA in producing retardation of amebocyte aggregation, it must be assumed that the aggregation promoting material in serum and AHS circumvents a physiological process, or processes, blocked by EDTA. Moreover, it appears that this process blocked by EDTA is probably an early event in aggregation rather than the initiation of aggregation. Three plausible explanations can be considered for the relationship between aggregation promoting material and EDTA in amebocyte aggregation:

(1.) EDTA could render amebocytes insensitive to aggregation promoting material present in preparations of amebocytes in EDTA. Addition of excess serum or AHS would then create an over-abundance of the aggregation promoting material plus small quantities of ions and competitively, or otherwise, override the EDTA inhibition. This explanation is, in part, contradicted by the observation that very dilute preparations of serum and AHS can cause rapid and complete aggregation of EDTA inhibited amebocytes.

(2.) EDTA could retard amebocyte aggregation by either preventing the formation of the aggregation promoting material, or directly inactivating the material itself. It appears that aggregation promoting material is released intact from amebocytes and the similar restorative properties of AHS and serum support this evidence. Then, EDTA must act intracellularly to prevent the formation of aggregation promoting material. However, amebocytes treated with EDTA contain active, aggregation promoting material. It, therefore, does not seem likely that EDTA produces inhibition of amebocyte aggregation by preventing the formation of the aggregation promoting material. On the other hand, when amebocytes in EDTA are added to serum (diluted 1 : 20) plus increasing concentrations of EDTA, the aggregation progressively decreases with increases in the EDTA concentration. Yet, after dialysis, serum treated with EDTA is fully active. It is possible that EDTA might directly inactivate the aggregation promoting material, but such inactivation is readily reversible.

(3.) EDTA could retard the release of the aggregation promoting material from amebocytes. Additional aggregation promoting material in the form of serum or AHS would then cause aggregation by initiating or enhancing release of more aggregation promoting material by the EDTA inhibited amebocytes. This explanation is supported not only by the isolation of aggregation promoting material from amebocytes treated with EDTA but also by the direct evidence shown in Figure 7.

Due to the preliminary nature of the evidence reported here, no one of these three explanations can be completely eliminated, but the hypothesis that EDTA retards amebocyte aggregation by preventing the release of the aggregation promoting material from amebocytes is, at present, the most appealing for several reasons.

Release of intracellular constituents by mammalian platelets is acknowledged as a prerequisite for irreversible platelet aggregation (Mills and Roberts, 1967), and from extensive studies employing chelating agents, it is generally agreed that calcium participates in the thrombin induced release reaction of platelets (Grette, 1962; Hovig, 1964; Zucker and Jerushalmy, 1967). More specifically, Kinlough-Rathbone and Mustard (1971), have reported that intraplatelet calcium is essential for the release reaction, but external calcium is required for the maintenance of intracellular calcium levels. It has been shown that aembocytes also release intracellular constituents during aggregation (Loeb and Bodansky, 1926; Levin and Bang, 1964a and 1964b; Dumont, Anderson and Winner, 1966). Furthermore, calcium is implicated in the release of intracellular consituents by a variety of cell types (see review by Stormorken, 1969). In light of the above information and the evidence presented in Figure 7, it is quite possible that EDTA retards amebocyte aggregation by interfering with the release of aggregation promoting material from amebocytes. In support of the possible release inhibiting role of EDTA, it is perhaps significant that colchicine which inhibits the release reaction of platelets (White, 1969) enhances EDTA inhibition of amebocyte aggregation, and N-ethylmaleimide which inhibits both amebocyte aggregation (Bryan, Robinson, Gilbert and Langdell, 1964) and the release reaction of platelets (Harrison, Emmons, and Mitchell, 1966) clearly prevents the release of aggregation promoting material from amebocytes (unpublished observations).

The presence of aggregation promoting material within hemostatic cells is by no means a novel concept. The early observations of Tait and Gunn (1918) indicated that a coagulating factor was present within the explosive corpuscles of the crayfish. Upon contact with foreign surfaces, these explosive cells released coagulating factor which in turn promoted plasma coagulation around the aggregating cells. In a review of studies on arthropod blood coagulation, Maluf (1939, p. 179) concluded that "clotting in most arthropods is probably evoked by a liberation of thrombin into the hemolymph as a consequence of the rupture of certain blood cells." Moreover, Born (1965) asserted that the transition between the adhesion of a few platelets at the specific site of injury and the formation of an effective platelet plug must involve some sort of chain amplification mechanism. Although a specific relationship has yet to be established, the release *in vivo* of such substances as ADP and vasoactive materials by aggregating platelets may be the mechanism which is responsible for thrombus formation (Mills, Robb and Roberts, 1968). Ultimate proof of the specific involvement of an aggregation promoting material in *Limulus* amebocyte aggregation awaits identification and characterization of this material. Furthermore, the specific site for EDTA inhibition of amebocyte aggregation remains a pressing question. Since there is no evidence which indicates that aggregation promoting material is consumed or inactivated during aggregation, a control mechanism must exist to limit aggregation to the site of injury. Most important, the mechanism which initiates the transformation of amebocytes from the circulating to the aggregating state remains completely elusive.

SUMMARY

1. The photometric method for measuring cell aggregation was adapted to study the aggregation of the amebocytes of the horseshoe crab, *Limulus polyphemus*.

2. At 15° C and a constant strring speed, amebocyte aggregation showed reproducible characteristics. Lowering the temperature below 15° C decreased both the rate and extent of aggregation but some slight aggregation still occurred at 0° C.

3. Aggregation was markedly retarded by buffered EDTA. The retardation of aggregation was dependent on the concentration of EDTA. Equimolar magnesium restored full aggregation to amebocytes in EDTA, whereas, similar concentrations of calcium caused only partial aggregation of EDTA treated amebocytes.

4. Dilute quantities of the serum supernatant of aggregated amebocytes and the supernatant of homogenates of amebocytes isolated at 0° C caused immediate aggregation of ameboyctes in EDTA. No significant aggregation was noted when EDTA treated amebocytes were added to the cell free plasma of hemolymph withdrawn at 0° C.

5. Serum or amebocyte homogenate supernatants did not require additional calcium and/or magnesium to induce aggregation of amebocytes in EDTA.

6. The aggregating activity of serum and amebocyte homogenate supernatants was essentially non-dialyzable and heat labile.

7. The data indicate the presence of an aggregation promoting material within amebocytes that is released from the cells during aggregation.

8. The possible relationship between this aggregation promoting material and EDTA induced aggregation inhibition is discussed.

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FORTNIGHTLY MOLTING AND REPRODUCTIVE CYCLES IN THE SAND-BEACH ISOPOD, *EXCIROLANA CHILTONI*

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The degree to which environmental variations affect selection for temporally organized activities is related to both the amplitude and predictability of the environmental cycles. The ecologist should expect to find well-developed temporal organization in the activities of species inhabiting highly variable but predictable environments (Enright, 1970). The wave-washed sand-beach habitat epitomizes this kind of situation. The unique characteristics of the sand-beach habitat, especially the steep physical gradients, which vary over a broad range depending on the stage of the tide and time of day, have led to a considerable interest in the ecology and behavior of its fauna. At any point on the beach, many aspects of the physical environment including wave turbulence, consistency of the substrate, temperature, salinity, pH, and the concentrations of oxygen and carbon dioxide (Bruce, 1928; Emery and Foster, 1948) show marked variations with time. Only a limited number of species of macrofauna have evolved the ability to tolerate these varying physical conditions.

The behavior of the sand-beach isopod, *Excirolana chiltoni*, is to a great degree governed by the tides. When the advancing wave wash of the incoming tide reaches the level on the high intertidal beach where these isopods are buried, they leave the sand to swim and forage in the wash zone. This period of swimming activity continues for approximately 2 hours past tide crest, after which the isopods once again burrow into the sand, which allows them to escape the ebbing tide and re-establish their position on the exposed beach until the next period of high water.

The survival value of temporally organized activities is particularly evident for this species, since its distributional zone lies farther shoreward than that of most intertidal sand-beach crustacea (Enright, 1961; Clark, 1969). *Excirolana chiltoni* is essentially a marine animal, requiring for its survival the periodic flooding of its habitat during high tide. These animals must maintain their distribution in an area of the beach where environmental gradients are steep and where an error in positioning of a few meters in the landward direction could place an individual out of the range of physically tolerable conditions. Since an individual could be incapacitated while molting, the question arises as to how this important activity might be coordinated to the tide cycle in a manner which allowed this species to maintain a favorable position in its habitat. This requirement provides the focal point for the present study.

Excirolana chiltoni stores calcium salts in localized concretions in its integument prior to ecdysis. The examination of preliminary collections of *Excirolana* taken from Scripps Beach (La Jolla, California) at intervals of 3 to 4 days during the summer of 1969, indicated that in some samples individuals with well-developed concretions were quite common while in other samples such individuals were either rare or entirely absent. Since the degree of development of these concretions parallels the molt cycle, this observation suggested that isopods tend to molt synchronously in the field. A more intensive and systematic program was undertaken in the summer of 1970 to look at the possibility of synchronous molting in greater detail, the results of which are presented here. In addition to the field study, molting frequency was monitored in a laboratory population held under non-tidal conditions in order to evaluate the possibility of an endogenous mechanism controlling the molt cycle.

METHODS

A 10 meter stretch of beach fronting on the Scripps Institution of Oceanography (La Jolla, California) was chosen as the study area. This beach is exposed to the open sea and has an average slope of 1–2% and a median sand grain diameter of 0.20 mm.

The backshore is eliminated by the presence of a concrete seawall; this makes Scripps Beach only slightly atypical, since the backshore is not well developed in adjacent areas where sandstone cliffs begin a few meters landward of the high-tide wash line. Wave wash reaches the seawall on the highest tides of the month, at least during the winter, when the beach is at its lowest level due to erosion by heavy surf. Wave wash rarely reaches the seawall during the summer when the beach is 50 to 100 cm higher as the result of sand deposition during periods of low wave action.

The tidal range on Scripps Beach at the time of new and full moon (spring tides) is considerably greater than the range at the first and last quarter (neap tides). The mean tide range is 3.6 feet but differences between high and low water can be as much as 6–8 feet during spring tides. During the lowest low tides, the exposed beach extends 150–200 meters from the sea wall, while during the highest low tides 50 meters or less of the beach may be exposed.

Excirolana chiltoni was collected from Scripps beach at 2-day intervals from June 17 to July 17 during the summer of 1970. On each sampling day a 50 meter transect line was laid out perpendicular to the seawall along the beach profile and a continuous trench 25 cm wide and 10 cm deep was dug parallel to this reference line. The trench, consisting of a series of contiguous 3-meter long sections, was sufficiently long to encompass the entire zonal range of the isopod band for that day. Sand samples from each 3-meter long section of the transect were sieved through a 0.5 mm mesh which retained even the smallest individuals in the population. Isopods collected in this manner were immediately killed with isopropyl alcohol and brought into the laboratory where they were preserved in buffered 5% formalin seawater.

RESULTS

Distribution

Figure 1 indicates changes in the distribution of *Excirolana* which were observed during the study period. The upper limit of the zonal band corresponds with the position of the washline of the preceding high tides on those occasions when the position of the high tide waterline was recorded (July 5 through July 17). The most obvious feature of these distributions is a movement of the population to progressively higher levels on the beach during weeks prior to both new and full moon (as the spring tides approached) and to lower levels on the beach during weeks following new and full moon (as the neap tides approached).

The position of the zonal band appears to have retained the same position relative to the high tide washline as the washline changed during the spring-neap cycle, resulting in a fortnightly shift in zonation. The amplitude of this movement is, however, somewhat different in the two cycles shown in Figure 1, although tide heights on corresponding days in each cycle were similar. This appears to be the result of the truncation of the distribution during the first cycle (June 19 to July 3) by the presence of Scripps seawall. The wave wash on spring tides struck the seawall which effectively stopped any further shoreward movement. This is somewhat atypical for this season, since in most years the level of the beach is sufficiently high by June to prevent wave wash from reaching the seawall even on the highest tides. An unusual period of high storms in the previous winter had eroded the beach to the extent that it had not yet reached its typical summer level by June 1970. The level of the beach, however, rose approximately

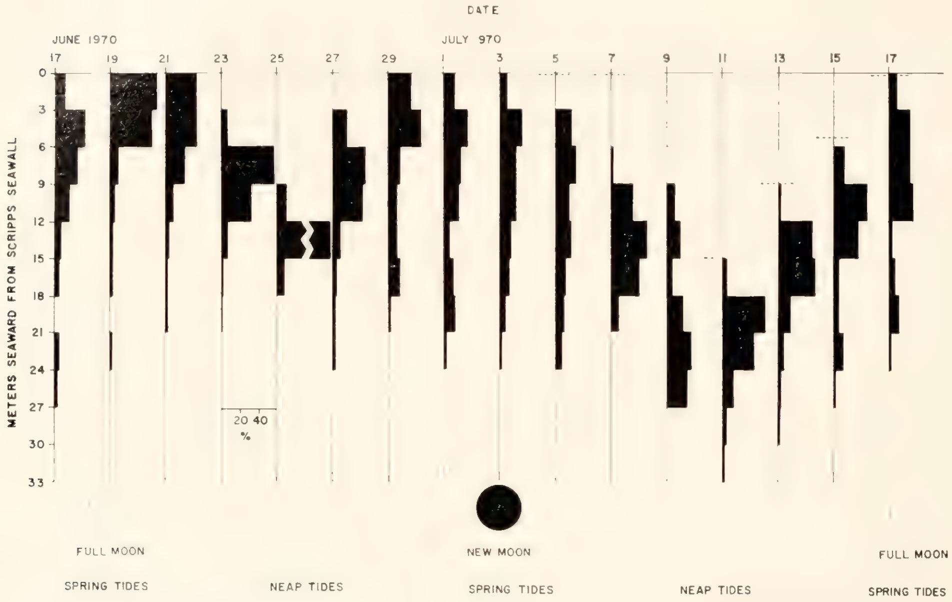

FIGURE 1. Semimonthly changes in the position of the *Excirolana* zone. The relative abundance of isopods in 3 meter sections of a continuous 25 cm wide transect across the zonal range, is indicated by each histogram. Transects were taken at two-day intervals on Scripps Beach. The width of the black bars indicates the relative abundance of isopods in each of the 3 meter long sections of each transect, expressed as a percentage of the total population. The dashed lines indicate the level of the beach reached by high tides during the interval between consecutive transects. This level was determined on July 5th to July 17th by observing the position on the beach where the previous trench had been obscured by wave wash.

½ meter from July 1 to July 9, which resulted in a seaward displacement of the distribution during the latter half of this study.

Similar fortnightly movements up and down the beach are a conspicuous feature of the distribution of a number of sand-beach animals, having been demonstrated for the bivalve *Donax gouldi* (Loren Haury, Scripps Institution of Oceanography, unpublished), and the cirolanid isopod *Eurydice pulchra* (Fish, 1970). They probably occur as well in the amphipod, *Synchelidium* sp. (Enright, 1961) and the anomuran crab *Emerita analoga* (Efford, 1965). While only the daily tidal component of migration has been looked at in the last two species, each assumes a zonal distribution on the exposed beach during low tide which is correlated with the amplitude of the previous high tide. A fortnightly migration is therefore implied, although time-series data over the spring-neap cycle were not actually collected.

Calcium storage in the integument

Two pairs of previously undescribed structures which store calcium carbonate and phosphate salts are present in *Excirolana chiltoni* (Figure 2). They are

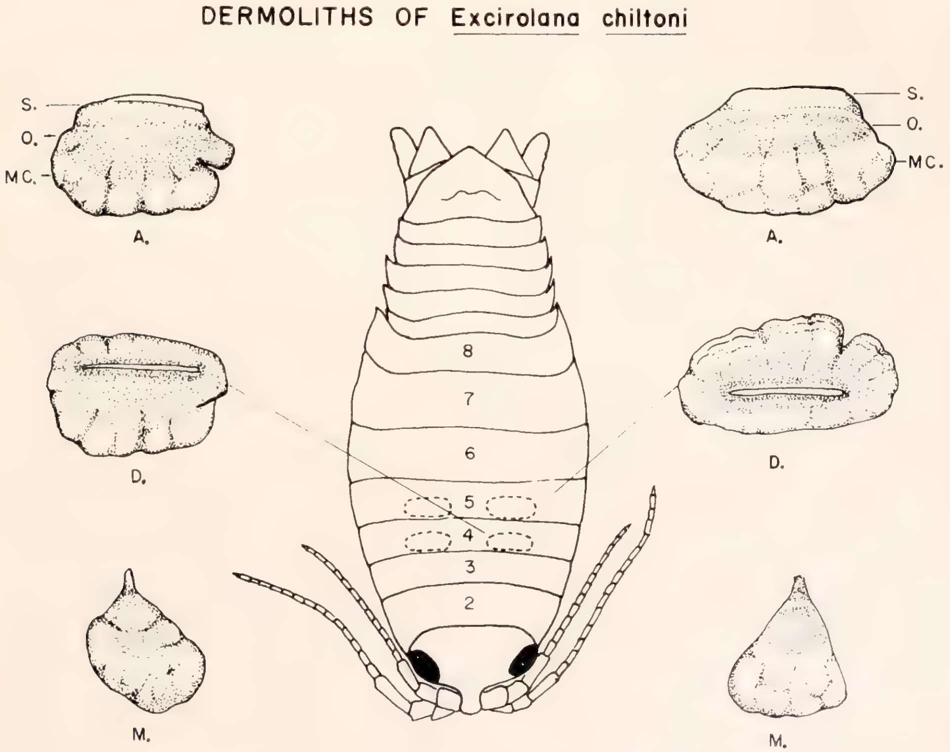

FIGURE 2. Sites of mineral storage (dermoliths) in the integument of *Excirolana chiltoni:* these structures can be seen through the translucent dorsal body wall; A., anterior view; D., dorsal view; M, median view; S., stalk area; O., opaque central core; MC., molded are area of calcium salts.

attached to and continuous with the anterior margins of the 4th and 5th thoracic segments along the dorsal surface. While they serve the same function as the gastroliths of the decapods, they differ in that there are two pairs rather than one, and that both are apparently derived from the tissues of the integument rather than the epithelium of the foregut. The storage structures, because they are derived from the mineralized layer of the integument, will be referred to as dermoliths.

Each dermolith is composed of three morphologically distinguishable parts. These are: (1) a broad dorsal stalk composed of material which is continuous with the mineralized layer of the integument, connecting to (2) an opaque central core, which is surrounded by (3) a large sculptured mass of calcium salts. The size of the dermoliths increases markedly prior to the molt, indicating that their primary function is the storage of mineral reserves to be used in hardening the post-ecdysial exoskeleton. There is a consistent difference in the shape of the anterior and posterior pairs of dermoliths. Dermoliths of the anterior set possess a prominent lateral projection while those of the posterior pair have a deep cleft on their trailing margins.

The dermoliths dissolve in dilute hydrochloric acid, except for the opaque central core which leaves a small residue. They produce gaseous bubbles, indicating the presence of calcium carbonate. X-ray diffraction patterns of material from 20 dermoliths failed to show any of the characteristic peaks of the known crystalline forms of calcium carbonate (calcite, aragonite or vaterite). A rather low diffuse peak was present, diffracting at an angle of about 30° which is characteristic of phosphate minerals. Calcium carbonate must, therefore, be present in an amorphous form. Prenant (1927) has found that only a few per cent of phosphate ions on a molar basis with calcium ions are sufficient to inhibit the crystallization of calcium carbonate; x-ray fluorometry indicates significant quantities of phosphorous in addition to calcium in the dermolith of *Excirolana* (Klapow, 1971). The mineral contents of fully developed dermoliths can be as much as 37.8% of the mineral salts in the entire exoskeleton.

Molting in *Excirolana chiltoni* takes place in two stages. After the dermoliths have reached their maximum size, a split develops in the exoskeleton between the 5th and 6th thoracic somites. The exosekeleton posterior to the split (the last 3 thoracic somites and abdomen) is then cast. The anterior exoskeleton is not cast immediately but follows approximately 25 hours later. During this intervening period, the new exosekelton on the posterior part of the isopod expands while the anterior portion of the isopod, still confined by the pre-molt exoskeleton, retains its original dimensions. An individual at this stage can be recognized by the break in profile at the junction of the 5th and 6th thoracic somities: the profile of the ventral lateral margin of the thoracic somites is straight during the intermolt but shows a distinct step-like expansion at the 6th thoracic segment once the posterior exoskeleton is cast.

During this half-molted stage, the mineral reserves in the dermoliths are absorbed into the blood, and the isopod assumes an opaque white aspect. At the same time that the dermoliths are being absorbed, re-calcification of the newly formed and fully expanded posterior exoskeleton is in progress (acidifying the posterior exoskeleton during the half-molted stage leads to the evolution of gaseous bubbles, indicating the presence of $CaCO_3$). Absorption and deposition occur simultaneously in different portions of the integument. This raises an interesting physiological question since these processes are generally considered to be under the control of blood-borne hormones which have a general effect throughout the body (Guyselman, 1953). The manner in which a widely distributed blood-borne substance produces a different response in separate parts of the integument remains an open question.

Following the absorption of the dermoliths, the anterior exoskeleton (head and first 5 thoracic somites) is shed and the isopod returns to its normal intermolt proportions. *Excirolana* retains its opaque white aspect until the remaining calcium salts, dissolved in the blood, are deposited in the anterior integument. On occasion, small portions of the dermoliths may not be completely absorbed and can be recognized on the exuvia of the anterior cast, demonstrating clearly that they are, in fact, derived from the integument.

A two stage molting process with a considerable delay between posterior and anterior casts has been reported in *Ligia* (Tait, 1917; Numanoi, 1934) *Oniscus* (Messner, 1966) and *Porcellio* (Messner, 1966). All of these isopod genera belong to the suborder Oniscoidea. *Excirolana* belongs to the suborder Flabellifera. The occurrence of this peculiar two-phase molt in two distinct suborders suggests that it may very well be characteristic of all the Isopoda. Schultz (1969) thought that this was, in fact, a general feature of molting in isopods, although he was undoubtedly mistaken when he stated that the anterior exoskeleton is shed first. In all of the cases cited above, the posterior portion of the exoskeleton is shed first.

Fortnightly molting and reproductive cycles: field data

The series of 25 cm wide continuous transects yielded on the average 293 isopods per collection (range 67–540 for 16 collections). Each specimen was examined and the following information recorded: (1) Total length, measured from the tip of the telson to the most anterior point on the head; (2) Stage of development; juveniles were classified as either 1st, 2nd, or 3rd stage mancas; older individuals were sexed; (3) Stage of the molt cycle as characterized by the development of the dermoliths; (4) The presence or absence of food in the gut; (5) The presence or absence of external parasites on the ventral thorax.

There are 3 manca stages in *Excirolana chiltoni*. The 1st and 2nd mancas lack the 8th pair of thoracic legs, which appear in a rudimentary condition in the 3rd manca stage and are fully developed in the post-manca stages. The 1st and 2nd manca stages which are morphologically similar were separated by size: specimens shorter than 2.5 mm were considered to be 1st stage while those that were longer than 2.5 mm were designated as 2nd stage. The size frequency distributions of 1st and 2nd stage mancas overlap somewhat, although not to the extent where the selection of the 2.5 mm cutoff point would result in a serious error. The half-molt, in transition from the 1st to 2nd manca stage is morphologically unique. It is the only half-molt stage which lacks any trace of the 8th pair of thoracic legs.

The dermoliths are not always present but undergo a cycle of accretion and absorption which parallels the molt. Their stage of development can, therefore,

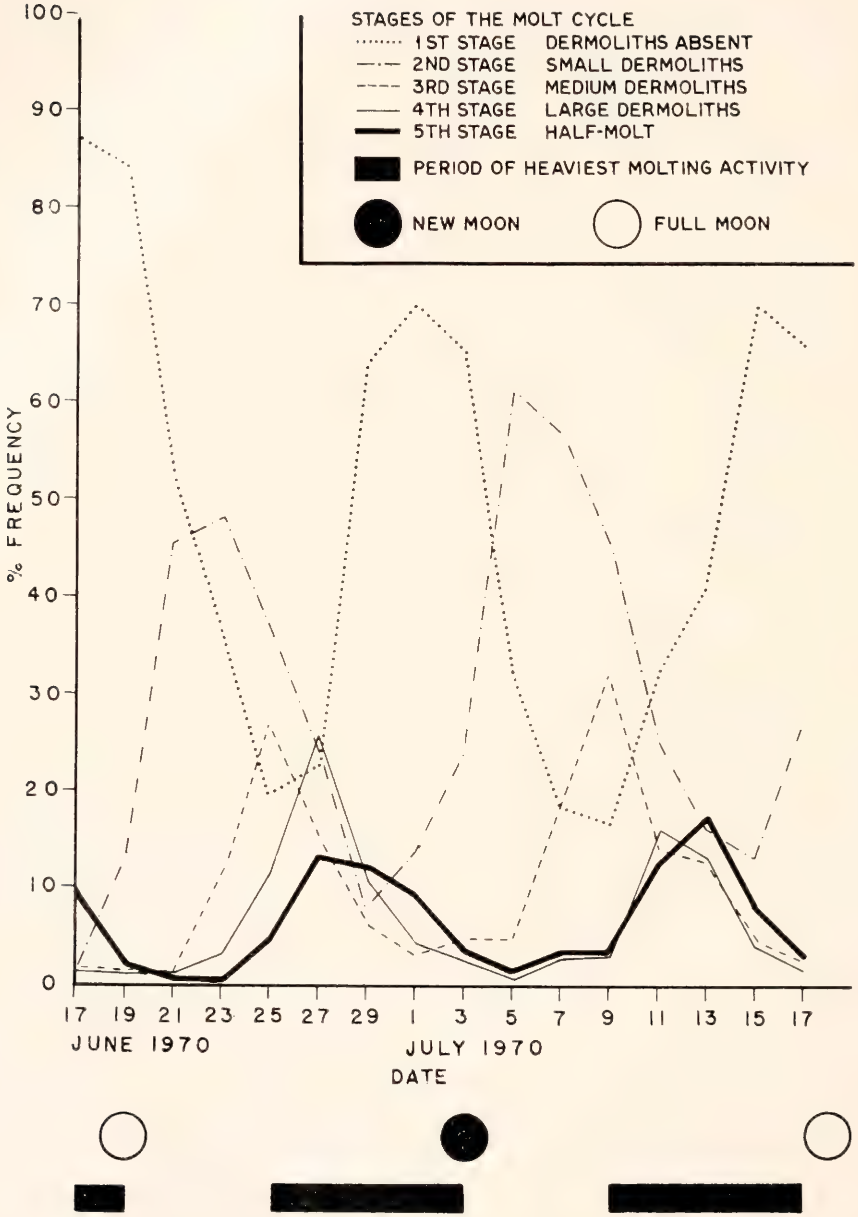

FIGURE 3. Relative abundance of the 5 stages of the molt cycle as a function of time. The half-molt stage represents isopods which are in the process of molting.

provide convenient indices for the molt cycle. The molt cycle was divided into 5 stages based on this character. The 1st intermolt stage lacks dermoliths entirely. This is followed by the 2nd stage (small dermolith stage) in which the broad stalk area of the dermolith is present. In stage 3, both stalk area and opaque central core are present (medium dermolith stage), while in stage 4 the dermoliths are fully developed with stalk area, opaque central core and the large molded area of calcium salts all present. The 5th stage is the half-molt individual which has shed the posterior exoskeleton, and is the stage in which the dermoliths are in the process of being absorbed. Previous schemes to classify the molt cycle of crustaceans have been based on histological changes in the integument (Travis, 1955, 1957). The important advantage in defining the molt cycle of *Excirolana* by the development of dermoliths is that this character can be assessed by direct examination, since the dermoliths are clearly visible through the translucent dorsal body wall in most specimens. Large opaque individuals were dissected to determine the degree of development of their dermoliths.

The presence or absence of external parasites on the ventral body surface was also recorded. These parasites are stalked ciliate protozoans which attach to the integument and are lost with each molt. Isopods which have just cast are free of parasites but become reinfested with time. The degree of infestation of the population can therefore be used as an indication of the time since the majority of individuals last molted.

Gravid females do not molt while they are carrying embryos (gestation lasts for approximately 2 to 3 months in *Excirolana chiltoni*). For this reason they are not included in the analysis of molting frequency in the field. Only 71 gravid females were found out of 4690 specimens examined. This constitutes only 1.5% of all collections so that their exclusion from the analysis is relatively unimportant.

Figure 3 shows the relative abundance of each of the 5 stages of the molt cycle as they vary with time. Note that there is a fortnightly variation in the relative abundance of each stage. Note also that there is a progression in the abundance of the stages; first the curve indicating the relative abundance of isopods without dermoliths peaks (June 17), followed a few days later by a peak in the abundance of isopods with small dermoliths (June 23), then medium dermoliths (June 25), then large dermoliths (June 27), and finally the half-molt stage. The same progression in the abundance of stages was repeated in the second half of the study so that two complete molt cycles are indicated during the month of field collections. These two facts, the fortnightly variation within each stage and the ordered progression in peaks of abundance of the 5 stages, indicate that molting in *Excirolana chiltoni* is synchronized to a fortnightly cycle. Most of the actual molting activity is confined to a week prior to either the new or full moon, as indicated by the higher relative abundance of half-molted isopods at these times. Peak molting activity occurs in the middle of these intervals, that is, 4 to 5 days before the spring tides.

The numbers of cast exoskeletons found in the field collections show a fortnightly periodicity as well. Of the 102 intact exuvia (either anterior or posterior casts) which were found in the field collections, 94 were recovered during the weeks prior to new or full moon while only 8 were found on the weeks following new or full moon.

Figure 4 provides additional evidence for a synchronized fortnightly molting cycle. The dashed curve represents the percentage of parasitized isopods in the population as a function of time. Note that the incidence of parasitism decreases as the population enters the period of heaviest molting activity and rises sharply during the weeks following the new or full moon, as individuals become reinfested with external parasites following the molt.

The percentage of isopods with food in their guts, indicated by the solid line in Figure 4 also shows a fortnightly variation. Two alternative hypotheses could account for this observation: there may be a fortnightly variation in the availability

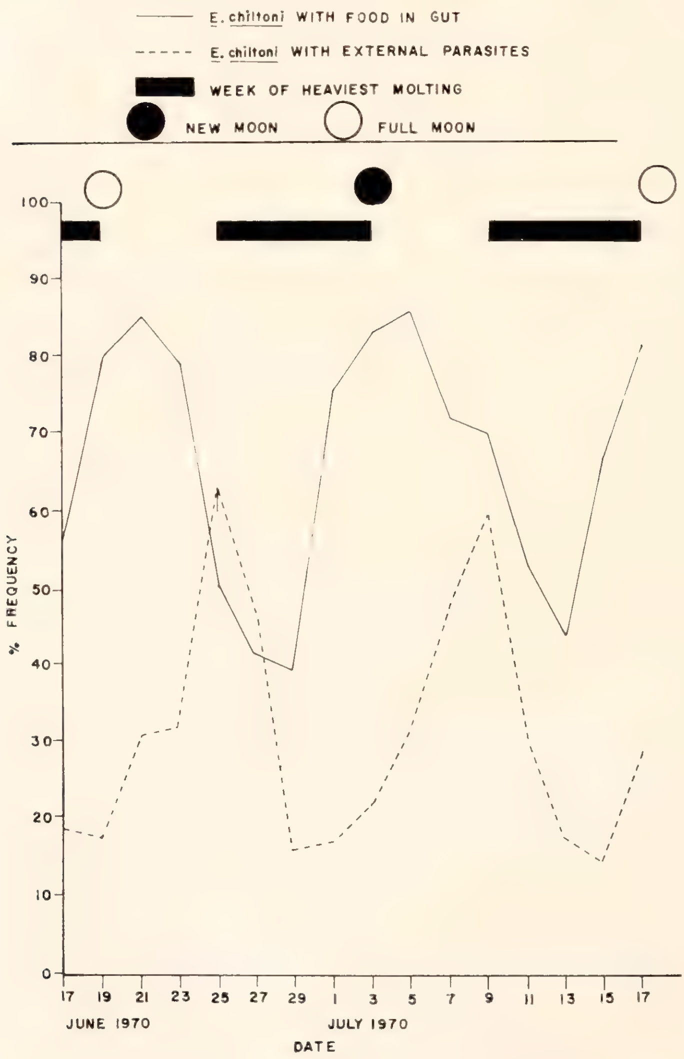

FIGURE 4. Relative abundance of feeding and parasitized isopods as a function of time.

of food in the habitat or there may be innate changes in feeding propensity, associated with the molt cycle. In the first case, periodic feeding could be the cause of molt synchrony; in the second case, it would be an incidental effect.

Fluctuations in the availability of food could arise by either of two processes. There might be a semimonthly variation in the actual concentration of food resources on the beach, or there could be a variable intake of food, due to fortnightly changes in illumination: *Excirolana* forages during the high tide, so that the light regime at which it feeds undergoes a regular fortnightly change as the time of peak tide progresses through the day-night cycle. If visual clues are important for the isopods to locate food items, the illumination cycle could lead to a fortnightly change in feeding activity.

If the first hypothesis is correct, then the percentage of feeding individuals in each of the 5 stages of the molt cycle should show the same fortnightly periodicity. The proportions of feeding individuals in each of the 5 stages should increase simultaneously when food becomes available, and decrease simultaneously when food once again becomes scarce. Kendall's concordance analysis (Table I) was employed to test this hypothesis. The percentage of isopods in each stage of the molt cycle with food in their gut for each of the 16 field collections is given in Table I. The 16 values were ranked across the rows for each stage and the columns of ranks summed. If there were a periodic availability of food, then in some collections all of the 5 stages should simultaneously show elevated feeding levels while in other collections there should be a simultaneous decline. This would lead to a marked departure of individual column rank sums from the average value of all column sums which is 34. In other words, there would be a high degree of correspondence in the percentage of feeding individuals for all stages of the molt cycle on each sampling day, which would lead to a significant probability for the Kendall concordance coefficient (Tate and Clelland, 1957, page 19). This is not the case (Kendall's concordance coefficient $= 0.156$, $0.90 > P > 0.75$) which suggests that there is no fortnightly cycle in the availability of food.

A runs test for periodicity (Wallis and Roberts, 1956, page 572) applied to each of the intermolt stages (stages 1 through 4) indicates a significant periodicity in the level of feeding activity for the 1st intermolt stage ($P = 0.001$), but not for the 2nd, 3rd, and 4th stages. The periodicity observed for the 1st intermolt stage is, however, a predictable consequence of the fact that it follows the half-molt stage which does not feed. The transition from a non-feeding to feeding condition in the 1st intermolt stage could account for periodicity in its level of feeding activity. The lack of concordance in the level of feeding activity for all stages seems more important for considering the possibility of a periodic availability of food than periodicity in the feeding level of any single stage and indicates that the probability of an isopod obtaining food is independent of whether the animal is molting in phase or out of phase with the rest of the population. Therefore, the availability of food in the habitat does not seem likely as a factor leading to synchronous growth and molting.

The feeding cycle which becomes evident when the entire population is considered (Fig. 4) appears to be the result of a decrease in the propensity of *Excirolana* to feed as it approaches the time of molt. This becomes clear when the average level of feeding activity for the 5 stages of the molt cycle is considered

TABLE I.

Concordance analysis by ranks of the percentage of feeding individuals in each of the 5 stages of the molt cycles; Kendall's concordance coefficient, W = 0.156 (0.90 > P > 0.75)

	June							July									Average of 16 values (%)
	17	19	21	23	25	27	29	1	3	5	7	9	11	13	15	17	
1st stage (dermoliths absent)																	
% feeding	66.1	82.3	88.4	91.2	75.3	61.4	49.4	91.2	93.7	85.4	78.2	70.0	66.6	58.8	76.3	87.0	76.2
Rank	4	10	13	14.5	7	3	1	14.5	16	11	9	6	5	2	8	12	
2nd stage (small demoliths)																	
% feeding	100.0	75.0	84.1	83.8	86.6	100.0	100.0	84.7	86.9	92.4	88.7	81.4	100.0	78.8	96.6	87.5	89.0
Rank	14.5	1	5	4	7	14.5	14.5	6	8	11	10	3	14.5	2	12	9	
3rd stage (medium dermoliths)																	
% feeding	0.0	100.0	75.0	38.4	13.2	17.8	0.0	30.0	16.0	40.0	39.1	63.1	33.3	40.0	24.0	16.7	34.1
Rank	1.5	16	15	10	3	5	1.5	8	4	12.5	11	14	9	12.5	7	6	
4th stage (large dermoliths)																	
% feeding	0.0	0.0	25.0	10.0	1.9	3.8	0.0	13.3	7.1	0.0	0.0	50.0	10.0	11.1	4.0	0.0	8.5
Rank	3.5	3.5	15	11.5	7	8	3.5	14	10	3.5	3.5	16	11.5	13	9	3.5	
5th stage (half-molt)																	
% feeding	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Rank	Excluded from analysis since this stage does not feed																
Rank sums	23.5	30.5	48.0	40.0	24.0	30.5	20.5	42.5	38.0	38.0	33.5	39.0	40.0	29.5	36.0	30.5	

(see last column in Table I). There is a clear drop in the level of feeding activity in the 3rd through 5th molt stages. The decrease in the percentage of feeding individuals in Figure 4 occurs about the time that these stages begin to make up a significant proportion of the population (see Fig. 3). In summary, the fortnightly feeding cycle appears to be an effect rather than the cause of synchronous molting.

The apparent sychronization of molting activity to the spring-neap cycle could perhaps be the result of a synchronous input of young. These might then develop at approximately the same rate and, at least initially, molt in synchrony. One would expect that differences in the growth rate of individual isopods should eventually lead to a deterioration of synchrony after several molts.

To examine this possibility, the frequency of occurrence of the 1st intermolt stage (isopods which lack dermoliths) was computed separately for the 1st manca, 2nd manca, 3rd manca and post-manca males and females. This single index of the molt cycle was chosen because it is, on the average, better represented in the samples than the other four stages. Estimates of the abundance of this stage are, therefore, subject to a smaller degree of sampling variability than the other less abundant stages. These data are shown in Figure 5. Note that molting for each of the developmental stages is synchronous and phased to the tides. There is no apparent loss in synchrony, even in the post-manca males and females. These latter stages have undergone at least 3 molts in the field. In fact, some individuals in the post-manca categories were born in the previous year (Klapow, 1971), and judging from their size had probably undergone several additional molts since birth. It appears, then, that development is continuously adjusted so that molting will at all times occur at the same phase of the spring-neap cycle.

This does not exclude the possibility of a synchronous input of young. Such a mechanism could account for initial molt synchrony but would not provide an adequate explanation for the maintenance of synchrony over several molts. A most interesting aspect of these data is, in fact, the apparent synchronization of the first molt following birth. The 1st stage mancas molt in phase with later developmental stages. This would suggest the possibility that the 1st stage mancas might have been born in synchrony.

Further evidence which supports the proposition that 1st stage mancas molt in synchrony is presented in Figure 6 (graph A). Graph A is a plot of the relative abundance of half-molt individuals in transition from the 1st to 2nd manca stage, expressed as a percentage of the 1st stage mancas. Synchronization is apparent. Most of the molting activity takes place on the weeks prior to new or full moon, as is the case in later developmental stages. If the 1st stage mancas were born continuously, there would have to be considerable variation in the duration of the intermolt in order to achieve synchrony in molting. If, on the other hand, the delivery of young as well as molting, were phased to the tides, this problem would not arise.

There is no way of identifying a newly released isopod with complete certainty. One might however expect a newborn individual to have the following characteristics. It would, of course, have to be a 1st stage manca. In addition it might not have been in the environment long enough to have fed or acquired external parasites. While dermoliths are present in the late embryonic stages, one might expect

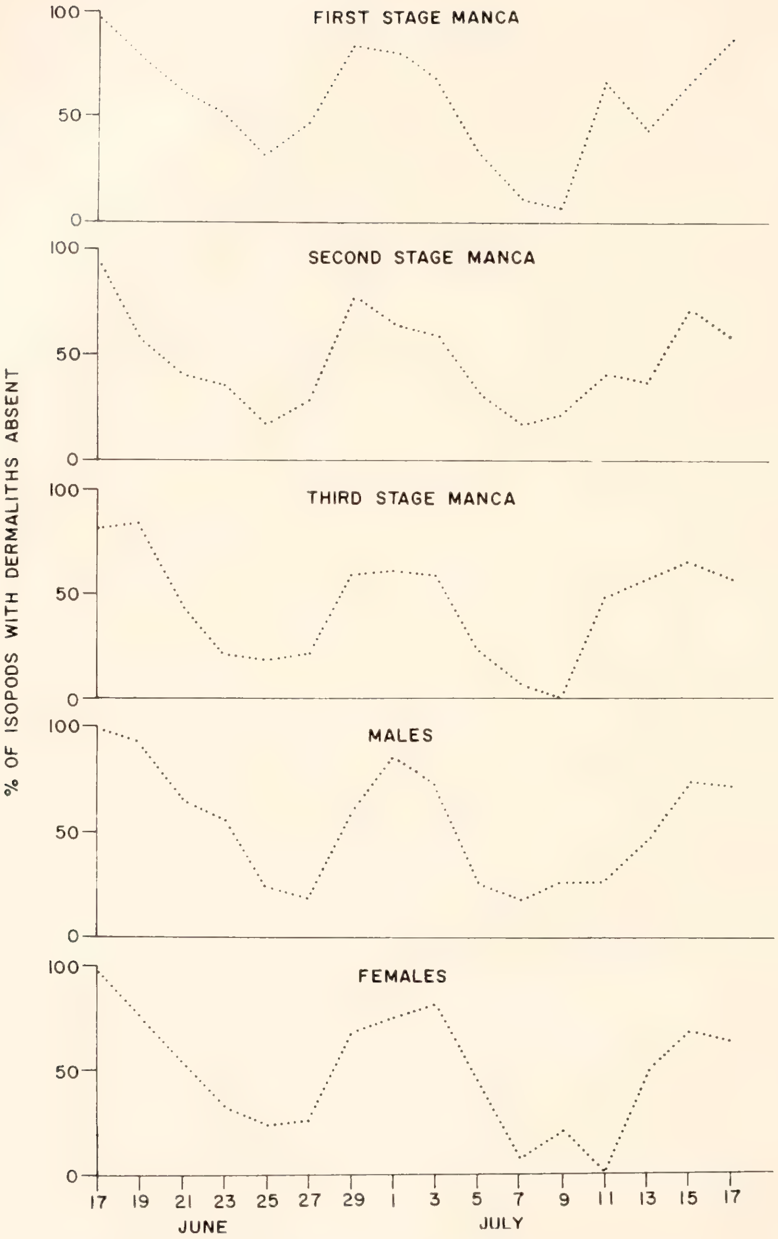

Figure. 5. Relative abundance of isopods in the 1st intermolt stage plotted separately for developmental stages.

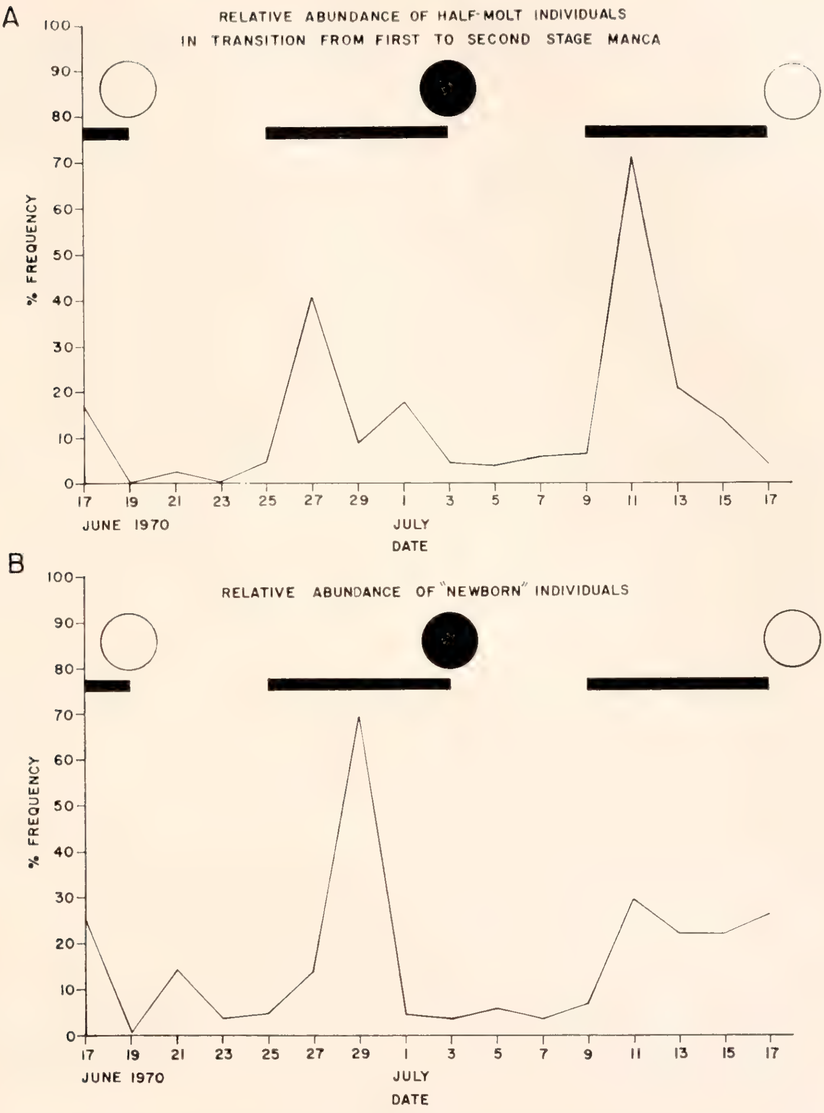

FIGURE 6. Synchronized molting and birth of 1st stage mancas. Open and closed circles indicate the day of new and full moon respectively. Black bars indicate the period of heaviest molting activity.

that they would be rapidly absorbed as the soft integument expands and hardens following birth. Dermoliths are completely absorbed in less than 25 hours in the half-molt stage and assuming that this rate of absorption applies to newly released isopods, they would then lack dermoliths shortly after birth. These three characteristics, the absence of dermoliths, of external parasites, and of food in the gut were employed to identify 1st stage mancas which were likely to have been recently released into the environment. Figure 6 (graph B) shows the relative abundance to these "newborn" individuals expressed as a percentage of all 1st stage mancas. If one accepts the criteria for identifying newborn isopods, it is quite clear that the release of young was synchronous and occurred at the same phase of the spring-neap cycle as molting in the population. An extremely well synchronized release of young occurred between June 27 and July 1 (Fig. 6, graph B) which presumbaly involved the same individuals which showed the highly synchronized molting activity which occurred approximately a fortnight later on July 9 through July 13 (Fig. 6, graph A). Thus newborn individuals are apparently released syncronously and molt two weeks later at the same phase of the tides during which they were born.

Molting in the laboratory

On June 23, 1970, 60 juvenile (manca stage) isopods were brought in from the field and their molting activities were monitored for 3 months. Each isopod was placed in a 50 ml vial which was filled with sand to a depth of 1 cm. A mesh

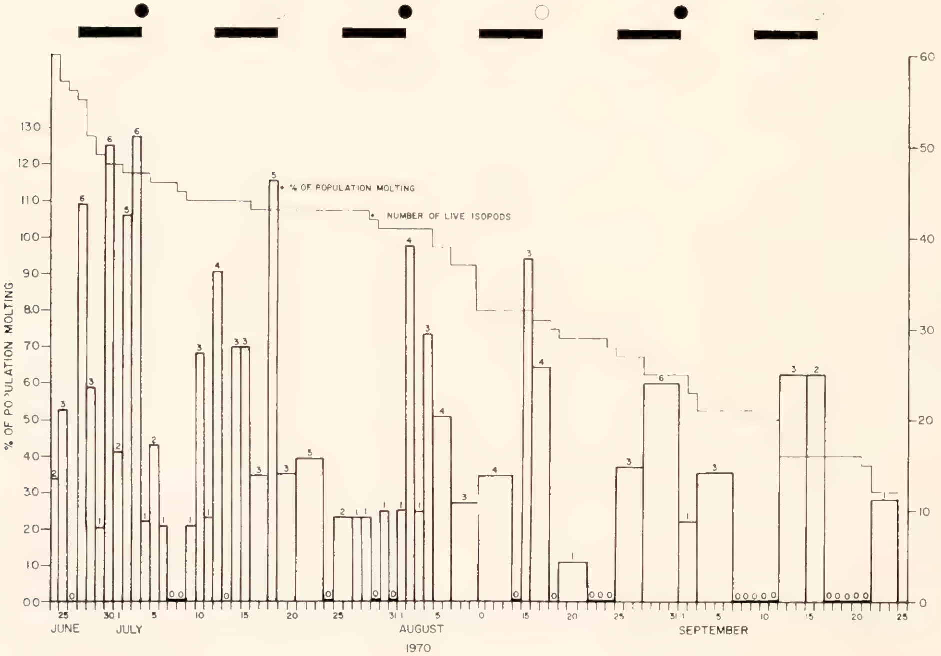

FIGURE 7. Molting frequency of *Excirolana chiltoni* in the laboratory. Molting frequency is indicated by the number of posterior casts recovered on the day of examination (number above each bar) which is expressed as a percentage of the isopods that were alive at that time. All mortality was assumed to have taken place on the first day following the last examination when the interval between examinations was greater than a day. When the time between examinations was greater than a single day the average daily molt frequency for that interval is shown. Open and filled circles indicate the days of full and new moon, respectively. Bars represent the expected time of heaviest molting activity in the field.

window was inserted in the vial above the sand level to allow water to circulate through the vial when it was placed on a water table in Scripps Aquarium. Light was kept at a constant dim level but no attempt was made to control temperature which varied considerably over the study interval (17.5° C to 22.8° C). Each vial was stocked at regular intervals with an excess of food in the form of pieces of *Thoracophelia mucronata*, a major component of *Excirolana*'s diet on Scripps Beach. The vials were examined for the presence of cast exoskeletons every day for the first 21 days of the study and at 1 to 4 day intervals thereafter, at irregular times of day.

Considerable mortality occurred during the course of this molting study; molting frequency for the population is therefore expressed as a percentage of live isopods on the day of observation (Fig. 7). A weak periodicity is apparent for the first few weeks of the study. Molting was common during the week prior to the new moon of July 3, declined during the following week and rose again on the week prior to the full moon of July 18. Beyond the first cycle, the times at which the population was checked were too irregular to permit firm conclusions about the maintenance of synchrony. The suggestion of continued synchrony (note that high values usually occurred on days of full and new moon) may be only coincidental.

TABLE II.

Molt intervals for Excirolana chiltoni *in the laboratory (all entries are in days)*

	2nd manca	3rd manca	1st post-manca	2nd post-manca
	26	25	21	30
	19	26	30	24
	20	17	23	42
	18	18	22	39
	25	17	32	39
	17	16	20	52
	19	25	24	29
	17	20	29	33
	19	24	23	29
	15	18	22	32
	17	19	26	24
	20	21	27	
		22	22	
		27	30	
		32	39	
			30	
			31	
			41	
			23	
			27	
			30	
Range	15–26	17–32	20–41	24–52
Mean interval	19.33	21.80	27.5	33.91
95% C.L. on mean interval	17.28–21.38	19.27–24.33	25.06–29.93	28.24–39.57

Table II lists the duration of developmental stages in the laboratory. Only those intervals which were known to an accuracy of one day are recorded. These data differ from field observations in two respects: the duration of each stage is significantly greater than the semimonthly tidal interval of 14.76 days; and there is a progressive lengthening of the duration of each instar from an average of 19.33 days for the 2nd manca to 33.91 days for the 2nd post-manca.

In the field, all instars molt at the same phase of the spring-neap cycle (Fig. 5) which means that the only molt intervals realized under field conditions must be either 14.76 days or some whole multiple of 14.76 days. If there was a strong endogenous rhythm controlling the molting cycle, one would expect that the duration of instars under non-tidal conditions in the laboratory would be a whole multiple of some basic period which approximates, though need not be precisely, 14.76 days. This was not observed: the duration of consecutive instars increased progressively and there were no clear modes at or near multiples of 14.76 days.

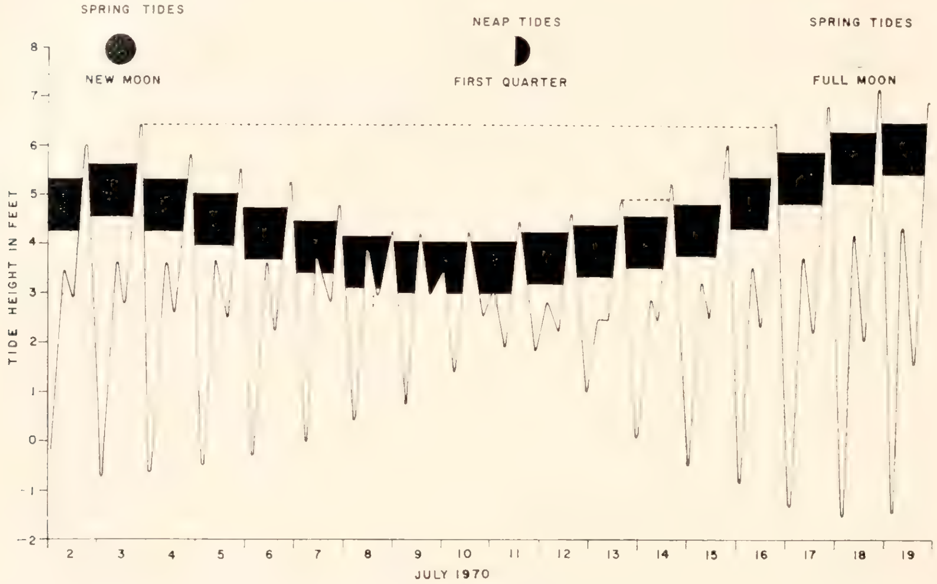

FIGURE 8. Idealized representation of the fortnightly movements of the isopod zone. Tide data are real but the position of the isopod zone is hypothetical. Blackened bars represent the position of the isopod band with reference to tide level (see Fig. 1 for field data substantiating the existence of the fortnightly movement of the zonal band). Dotted lines illustrate the fate of isopods which are stranded at the edge of the wave wash at high tide. Isopods stranded during the transition from spring to neap tides might be marooned above the water line for as long as 2 weeks, while animals stranded during the transitions from neap to spring tides would be reached by the high tide on the following day.

DISCUSSION

Excirolana shows fortnightly rhythmicity not only in the release of the young, but also in locomotor activity (Heusner and Enright, 1966), feeding, molting and the extent of parasitism. The question therefore arises: which of these, if any, is the primary rhythmicity, and which of these rhythms is of major ecological advantage to the organism? In brief, what is the adaptive significance of these phenomena?

Fortnightly cycles which have been previously studied deal primarily with reproduction. Synchronization of reproduction to the lunar cycle is particularly common among species with external fertilization which shed their gametes free into the environment. The first well documented example of lunar spawning and still one of the most dramatic cases is found in the Palolo worm, *Eunice viridis*, of the South Pacific Islands. Field observations on lunar spawning cycles are by no means confined to marine annelids, although these make up the bulk of well documented cases in the older literature. Korringa (1947, 1957), in his extensive surveys of the literature, found evidence for lunar spawning cycles in marine coelenterates, echinoderms, crustaceans, insects, and vertebrates. His reviews, while comprehensive in scope, did not involve critical evaluation of the claims, so that while there is reason to believe that lunar reproductive cycles are widespread, there are still only a few well established cases. One of these is the spawning cycle of *Leuresthes tenuis*, a small offshore fish which runs up on the beaches of Southern California and Mexico to spawn shortly after tide crest, over a period of 3 or 4 days beginning a day or two after new or full moon (Walker, 1949). The adaptive value of reproductive synchrony is obvious for species with external fertilization: by restricting the liberation of gametes to a particular time and place, the probability of successful fertilization is much enhanced.

Synchronous mating can also be beneficial for species with internal fertilization where physical contact between the sexes is required. In many crustaceans, impregnation of the female is only possible during a short interval which follows the molt. In the marine isopods, *Idotea emarginata* (Naylor, 1968), and *Idotea neglecta* (Kjennerud, 1950) insemination takes place as the female molts, as is the case with *Excirolana*. The availability of many receptive females, as a consequence of the synchronized molt, might enhance the incidence of successful matings by increasing the sexual responsiveness of males at this time. It is generally recognized that for those species where sexual receptiveness is intermittent, an appropriate state of behavioral and physiological readiness must be simultaneously achieved in both sexes if successful reproduction is to follow. Wheeler's (1937) study of the shrimp *Anchistioides antiguensis*, is directly pertinent for considering the possibility of synchronous reproductive activity in *Excirolana*. He found that this prawn swarms at the surface in the field on nights following the new moon. In addition he found what may have been a lunar rhythm in molt frequency in the laboratory. His data on molting are not very extensive (only a single cycle was observed) and the supposed lunar cycle is not particularly clear, but the possibility exists that there might be a causal relation between swarming in the field and the molting cycle which Wheeler observed in the laboratory, although he did not believe that this was the case.

Synchronius mating can convey other benefits in addition to increasing the likelihood of successful contacts between the sexes. Synchronous mating in some instances is required to facilitate the development of embryonic or juvenile stages in coordination with the tides so that their liberation will occur at a time when environmental conditions are most suitable. According to Hagen (1970) the necessity of releasing planktonic larvae at spring tide has apparently led to synchronous courtship and mating in the fiddler crabs *Uca annulipes*, and *Uca triangularis*. Courtship displays are most intense a few days before new or full moon, which is followed by insemination around the time of new or full moon and the planktonic larvae are released two weeks later on the spring tides. In the grunion *Leuresthes tenuis*, eggs which are deposited on the high beach during spring tides are incubated in the warm sand for approximately two weeks. The larval fish emerge from the egg capsule when they are wet by the spring tides of the next cycle. In all of the above cases the release of young takes place a fortnight after fertilization. *Excirolana chiltoni*, however, incubates its brood for approximately 2 to 3 months. The need for synchronous initiation of development is, therefore, not immediately apparent since the release of young at a particular phase of the spring-neap cycle could be accomplished by adjusting the duration of embryonic development by only a few days, that is, by a small fraction of the total gestation period. The synchronous molting and insemination of mature females which might possibly aid in achieving a synchronous release of young 2 to 3 months later, does not account for the fact that pre-reproductive isopods molt in synchrony as well.

Both of the hypotheses suggested up to this point have dealt with only a part of the total phenomena. An adequate hypothesis would have to account for the following facts: the release of young, as well as the molting of every developmental stage, is synchronized to the spring-neap cycle, and the phase of these activities corresponds to the transition from neap to spring tides (a time when the amplitude of the tide is increasing on each consecutive day). Figure 8 illustrates a hypothesis which provides a unified basis for interpreting the ecological significance for both the synchrony and phase of these processes. *Excirolana chiltoni* maintains a relatively constant position on the beach with reference to the high tide wash line over the spring-neap cycle. Isopods burrow down into the sand and escape the falling tide at a fixed time past tide crest (Klapow, 1971). This behavior will assure that *Excirolana* is washed at high water at least once each day. Two requirements would seem essential for maintaining this distribution. The first would be a precise sense of timing to allow the isopods to escape the receding tide at a level of the beach which will be reached by the next period of high water (Klapow, 1972). A second requirement would seem to be the continuous maintenance of swimming and burrowing ability since an incapacitated isopod might be carried to the limits of the wave wash in the same fashion that debris is washed up on the beach. Chandrashekaren (1965) observed that the mole crab, *Emerita asiatica*, when in a quiescent state following the molt, is carried up the beach to the limits of the wave wash. There is no reason to suspect that this would not happen to *Excirolana chiltoni* as well.

If an isopod were stranded at the upper limits of the wave wash at peak tide, its fate would depend to a great extent on when this happened in the spring-neap cycle. If it were stranded at peak tide during the transition from spring to neap tides, it could be marooned on the high beach, above the reach of the wave wash, for as long as two weeks. If, on the other hand, this were to happen during the transition from neap to spring tides, the consequences would be far less severe. An individual stranded at the upper limit of the wave wash at this time would be reached by the high tide on the following day and could reestablish an appropriate position in its habitat on the very next cycle.

The principal assumption underlying this hypothesis is that *Excirolana* is less able to maintain position in the wave wash during its molt, because of reduced swimming and/or burrowing ability. One expectation deriving from this interpretation is that molting individuals might have a distribution higher on the beach than nonmolting individuals. The field data were examined for indications of such a trend. In those cases in which statistically significant differences in distribution were present, molting individuals were, indeed, somewhat farther landward than nonmolting, but in most sampling series, no signficant differences in the distributions of these two classes were detectable. Hence, the data do not provide strong support for the proposed interpretation.

Other data (Klapow, 1971) indicate that heavy storm waves can occasionally lead to major mortality in the population, and it is primarily at such times that molting individuals would be expected to be particularly vulnerable. During the present sampling program, surf conditions were rather calm (average swell of 1.9 feet); hence, the lack of strong support for the stranding hypothesis in the available data may indicate only that the synchronization of molting is an adaptation to sporadically occurring conditions which did not prevail during the sampling interval.

In summary, the stranding hypothesis, although not completely substantiated by the data, provides a unifying framework for the interpretation of *Excirolana's* fortnightly rhythms. The ecological advantage in synchronizing molting to the rising series of tides is clear and would seem to be the primary adaptation; other fortnightly rhythms appear to arise primarily as a means of achieving molt synchrony or merely as incidental consequences of the molt cycle. For example, the synchronous release of young facilitates synchronization of the first molt following birth to the appropriate phase of the tidal cycle while the other rhythms (*i.e.* feeding and parasitism) appear to be incidental consequences of the molt cycle.

The possibility exists that synchronous birth might constitute an adaptation in its own right, since newborn isopods with soft exoskeletons might also be subject to a high risk of stranding. While the data which suggest that the release of young at a particular phase of the spring-neap cycle (Fig. 6) involve indirect evidence, this conclusion is rendered more plausible by the unique reproductive morphology of this species which allows it to control the time of birth. In typical free-living isopods, the young are brooded in an external pouch, out of which they can crawl or swim after they have reached an appropriate stage of maturity. In contrast, *Excirolana chiltoni* is ovoviviparous (Klapow, 1970); the female retains her embryos within sacs which are completely sealed off from the external environment up to the time of birth. The young cannot escape until external openings to the chambers in which they lie develop on the ventral surface of the gravid female. Even after the development of these openings the embryos are not free to leave. Birth is apparently accomplished by an increase in the fluid volume of the female's hemocoel, which exerts pressure on the sacs in which the embryos lie, causing them to contract and eventually evert through the openings. The timing of birth is, therefore, activated by physiological processes occurring in the gravid female.

The storage of calcium reserves has obvious benefits for terrestrial and fresh water crustaceans, where this element is often depleted in the environment. It is, however, more difficult to develop a similar argument for the storage of calcium reserves in marine forms, since calcium is a major component of the dissolved salts in sea water (420 mg per liter). Therefore, one would not expect that calcification of the post-ecdysial integument to depend very heavily on an internal supply. In fact, the major part of the integumental calcium of marine decapods is absorbed from the external medium following ecdysis (Drach, 1939; Hecht, 1914; Needham, 1954; Robertson, 1937; Travis, 1955; Lafon, 1948).

It has, however, been reported by a number of authors that prior storage of mineral reserves leads to a more rapid hardening of the exoskeleton following ecdysis, and perhaps this is the major ecological advantage for storing mineral reserves in *Excirolana*. Paul and Sharpe (1916) found that of the three genera of decapods which they studied (*Cancer, Lithoides* and *Homarus*), *Cancer pagurus* stores concretions of calcium phosphates in the hepatopancreas to the highest degree and as a consequence post-ecdysial hardening of the exoskeleton is more rapid than in the other species studied. An interesting observation bearing on this point was made by Numanoi (1939) in his study of the shore crabs *Sesarma haematoceir* and *Sesarma dehaani*. The two species differ in that *S. haematocheir* produces gastroliths (concretions of calcium carbonate formed by the gastric epithelium) prior to molting while *S. dehaani* does not. Both species seek refuge in shallow pools at the time of ecdysis; *S. haematocheir* takes only two days to complete hardening of the exoskeleton, while *S. dehaani* requires nearly twice as long. Two lines of evidence support the idea that stranding on the high beach poses a potential hazard for *Excirolana:* (1) the synchronization of molting and birth to the weeks of rising high tides and (2) the storage of mineral reserves which presumably decrease the interval of reduced locomotor function following the molt.

The fact that a fortnightly molting rhythm was not particularly evident under non-tidal conditions in the laboratory argues against an endogenous controlling mechanism but does not completely exclude this possibility. It is generally recognized that the persistence of endogenous rhythms under constant conditions depends to a great extent on the particular conditions under which the organisms are kept (Bünning, 1967). The considerable mortality of isopods during the study (Fig. 7) suggests that the conditions to which they were subjected were less than ideal. The major source of mortality during the study resulted from clogging of the mesh screens which restricted the circulation of water through the vials. The high degree of variability in the duration of instars (see ranges in Table II) as well as the longer than fortnightly interval between molts might be artifacts of the experimental conditions in which stresses imposed by restricted circulation through the small vials and the availability of only a single food item (*Thoracophelia mucronata*) might have interferred with the normal developmental processes. *Excirolana chiltoni* shows clear monthly and semimonthly rhythms in swimming activity under constant laboratory conditions which persist for several cycles when the animals are kept in large containers (Heusner and Enright, 1966; Klapow, 1971; Enright, 1972) so that an endogenous timing mechanism with an appropriate period is at least available to this species. Furthermore, the fact that under field conditions the accumulation of mineral reserves takes place in anticipation of the actual molt (Fig. 3) indicates that at least some measure of internal timing is involved. Nevertheless, the possibility of exogenous influences cannot be excluded; perhaps periodic exposure on the high beach is necessary to maintain and accelerate the molt cycle.

I take pleasure in acknowledging the generous assistance and advice of Dr. J. T. Enright, Dr. E. W. Fager and Dr. W. A. Newman which they offered at all stages of this research. This research was supported by National Science Foundation Grant, GB 5471.

SUMMARY

1. The isopod, *Excirolana chiltoni* is found buried in the sand in a narrow zonal band on the high intertidal beach during low tide and emerges only during high tide to swim and forage in the wave wash. The position of the zonal band during low tides bears a constant relationship to the level of the water line during the preceding high tide. Because of this relationship the isopod zone moves up and down the beach on a fortnightly schedule which corresponds to the semimonthly variation in tide amplitudes (the spring-neap cycle).

2. *Excirolana chiltoni* stores considerable quantities of mineral prior to ecdysis in localized concretions of the integument (dermoliths).

3. Molting takes place in two steps. First the posterior portion of the isopod including the abdomen and last 3 thoracic segments is cast which is followed by the casting of the anterior portion of the animal. Isopods which are in the process of molting can be easily recognized since the newly exposed posterior exoskeleton is fully expanded before the anterior cast is made.

4. The development of dermoliths and the relative abundance of half-molted individuals were used to determine the frequence of molting in a field population which was sampled at 2 day intervals for a period of a month. These data indicate that molting is largely if not entirely restricted to the weeks preceding new or full moon when the amplitude of the tides is increasing.

5. *Excirolana chiltoni* also shows fortnightly periodicities in feeding and the degree to which it is infested with external parasites.

6. Indirect evidence suggests that the release of young also follows a fortnightly schedule and is most intense at the same phase of the spring-neap cycle that the population molts. Females retain their broods internally and might therefore be capable of controlling the timing of birth with considerable precision.

7. Synchronous molting was not as apparent in populations which were held under non-tidal conditions in the laboratory.

8. Synchronization of molting and birth to the ascending series of high tides and the storage of mineral reserves prior to ecdysis are interpreted as adaptations which serve to decrease the probability of an isopod being marooned above the waterline for extended periods of time where it would be subject to a high risk of death by desiccation.

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INNERVATION OF THE LATERAL CILIA IN THE MUSSEL, *MYTILUS EDULIS* L.

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The pumping of sea water by the mussel, *Mytilus edulis*, is accomplished primarily by the beating of the cilia on the lateral epithelium of the gill filaments (Wallengren, 1905). The possibility that this process is under nervous control has been demonstrated by severing the branchial nerve and depressing the rate of beating of the lateral cilia (Aiello, 1960).

Nests of small branches of unmyelinated nerves have been found under the frontal, lateral, and postlateral cells of the fresh water mussel, *Elliptio complanatus* (Satir and Giulula, 1970). Nerve fibers of *Mytilus* were observed (Aiello and Guideri, 1965) running between the base of the lateral ciliated cells and the supporting rod. The structural details of the innervation apparatus were not clarified in this report.

The present work concerns observations on the fine structure of the branchial nerve innervating the gill of *Mytilus*.

MATERIAL AND METHODS

All observations were made on the mussel *Mytilus edulis*. These mussels were collected from Long Island Sound and kept for a period of two to four weeks in a Dayno Ocean Aquarium at 5° C, pH 7.5–8.0, density 1.025. Before each experiment the mussels were placed in finger bowls at room temperature for two hours. The posterior adductor muscle was cut and preparations containing the visceral ganglion, branchial nerve and posterior half of the gill from the left or right side of the mussel were isolated and quickly immersed in 5 milliliters of 10.0 per cent glutaraldehyde in 0.2 molar *S*-collidine (pH 11.8) buffer for four hours. While in the fixative, one millimeter sections were made along the dorsal margin of the gill axis in a posterior direction. These sections were oriented with the dorsal margin facing upwards or displaced laterally in a counter clock-wise direction. This was followed by two 15 minute buffer rinses and post fixation in 2 per cent osmium tetroxide in the same buffer for two hours. The tissue was dehydrated with ethanol and embedded in Epon in the usual manner.

Semi-thin sections (approximately 0.5 microns) were cut with a glass knife and were stained with methylene blue-basic fuchsin. Although this method is of general utility, it is especially useful for peripheral nerve and blood vessels.

Thin sections were cut with a diamond knife on a Model MT-2 Porter Blum ultramicrotome (Sorvall) and were stained with 5 per cent uranyl acetate for 30 minutes and post-stained with lead citrate for 5 minutes. The sections were examined at 50 kV with a RCA-EMU-3B or Philips-EM-200 electron microscope.

The localization of serotonin (5-HT) in the branchial nerve from *Mytilus* was accomplished by means of the fluorescence-histochemical method (Falck and Owman, 1965). Excised gills were frozen in Freon 22, cooled by liquid nitrogen and then lyophilized for two days in a Speedivac-Pearse tissue dryer at 40° C and 10^{-4} torr. Next, the nerves were exposed to paraformaldehyde vapor (relative humidity 70 and 90 per cent at 80° C) for one to two hours, respectively, and embedded under vacuum in paraffin wax. Sections were examined on a Leitz fluorescence microscope. The standard Aristophot-Ortholux microscope was fitted with an HBO mercury lamp, a dark field condenser, a BG12 excitor filter and a 490 mμ barrier filter.

Some of the molluscs prepared for fluorescence were treated with intramuscular injections of reserpine in a dosage of 50 mg/kg mollusc or nialamide (a monoamine oxidase inhibitor) in a dosage of 300 mg/kg mollusc.

Specimens exhibiting specific fluorescence were treated with 0.5 per cent sodium borohydride. The specific fluorescence was restored by re-exposure to paraformaldehyde vapor.

OBSERVATIONS

General organization of the gill

In order to understand ciliary activity of the gills of lamellibranch molluscs, it is necessary to review, briefly, the organization of the nervous system and the gills in *Mytilus* (Field, 1922; Gray, 1924, 1929; Lucas, 1931a, 1931b, 1932).

In cross section each of the two gills appears in the form of a narrow "W" suspended from the dorsal margin of the mussel. The inner and outer surfaces (called lamellae) are made up of a large number of gill filaments running lengthwise in the lamellae. An afferent branchial blood vessel is found within the dorsal margin of each gill. It is within the lumen of this blood vessel that the branchial nerve is found. This nerve, which arises from a visceral ganglion, runs obliquely downward and backward to enter the dorsal margin of the gill. It is easily discernible as a distinct bundle staining faintly blue with methylene blue-basic fuchsin. In its course through the gill axis it distributes its fibers to the gill filaments. These nerve fibers enter the gill filament between the supporting rod and ciliated abfrontal cells. A single nerve bundle lies adjacent to the supporting rod of a gill filament (Fig. 1). The supporting rods contain no nerve fibers.

Ultrastructure of the innervation of the gill

Clusters of nerve cells leave the visceral ganglion and accompany the branchial nerve in its course to the gill epithelium. They are distributed throughout the branchial nerve and contribute to this nerve. These cells are unipolar and approximately oval in shape, measuring about 26 μ in length and from 10 to 17 μ in width. The contour of the nucleus is similar to that of the cell. The perikaryon contain granules which occasionally are so numerous that they displace the nucleus (Fig. 2). These granules may contain crystals and often have myelin figures and other membranous components in their interior. Remnants of degenerating organelles are also incorporated within the granules. The perikaryon contains several

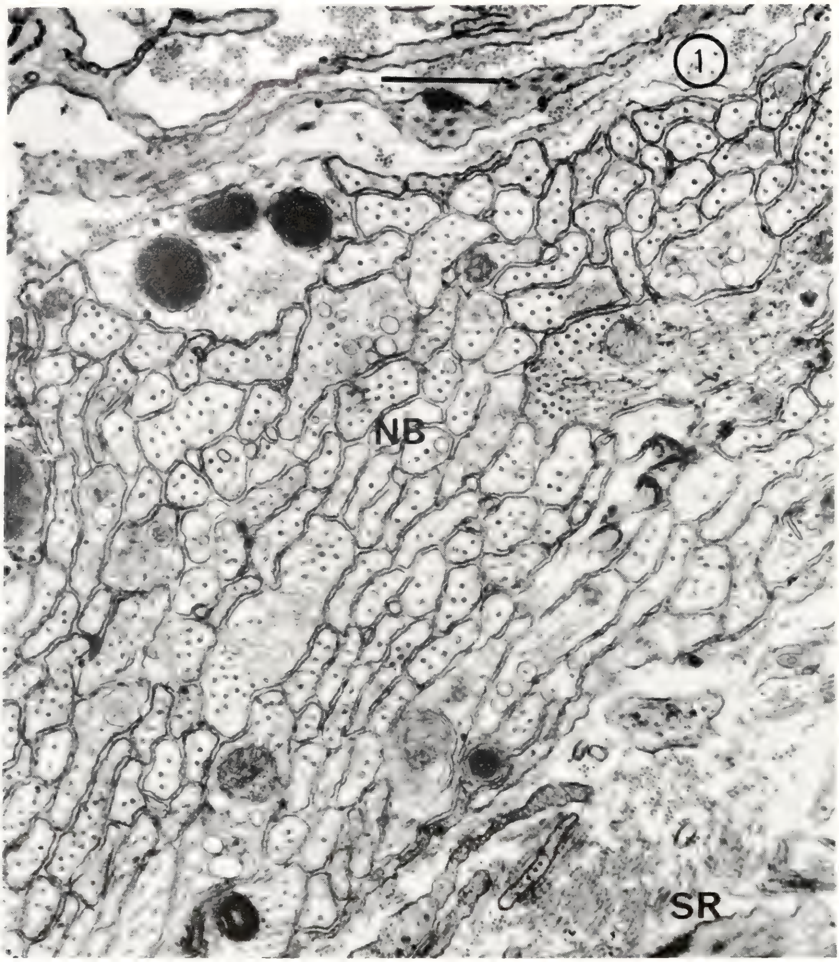


FIGURE 1. An electron micrograph of a nerve bundle (NB) in the afferent branchial vein. This bundle is shown adjacent to the supporting rod (SR) of the gill filament; scale = 0.2 μ

parallel arrays of obviously polarized cisternae of Golgi complexes. Membrane-bounded vesicles of various dimensions containing moderately stained material are commonly associated with these Golgi fields. These dense core vesicles arise by accumulation of products within the Golgi cisternae. Clear vesicles are also seen in the perikaryon, measuring about 700 Å. Discrete structures, occasionally appearing within the perikaryon, resemble small clusters of glycogen granules. Mitochondria are seen throughout the cytoplasm and on rare occasions contain crystalline inclusions. The granular endoplasmic reticulum is predominantly in the form of parallel cisternae with long profiles forming concentric whorls. The neural lamella that surrounds the nerve is composed of two elements, a finely fibrillar material and coarse striated fibers, presumably of a collagenous nature. The latter are predominantly oriented parallel to the axis of the nerve.

A nerve is found below the postlateral non-ciliated cell, which is just posterior to the ciliated lateral cell. The former cell type is easily distinguishable by the presence of large cytoplasmic granules. Both cells rest on a basal lamina. The ciliated lateral cell receives nerve fibers of the branchial nerve that pass through the basal lamina (Fig. 3). Most of the nerve fibers are found under a single cell, with the neighboring cells connected to it by intercellular bridges with cytoplasmic

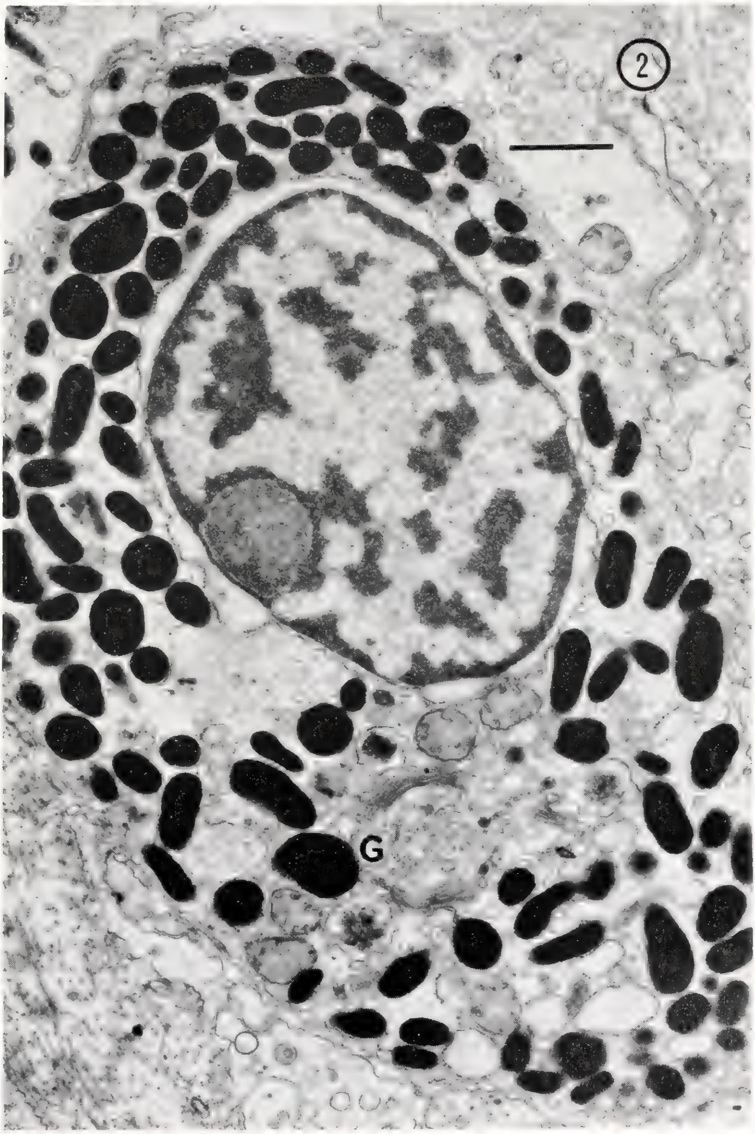


FIGURE 2. Perikaryon of a nerve cell containing electron dense granules. Stacks of cisternae of Golgi complex (G) are present in lower portion of cell. Membrane-bounded sesicles are found within the Golgi region; scale = 1.0 μ

continuity. The nerve fibers are about 1 μ in diameter and unmyelinated. They contain glycogen-like granules, few mitochondria and many rounded and oval non-granulated vesicles up to 650 Å in diameter. In these nerve fibers some vesicles contain a dense granular material. Single nerve fibers rich in vesicles, both granular and non-granular, have been found in close contact with a lateral cell. While vertebrate-type synapses were not seen, nerve endings have been found which possess some distinguishing morphological features. A space of about 200 Å separates the plasma membranes, and the nerve fiber is often contained in a gutter in the lateral cell surface with the plasma membrane lining the gutter and closely

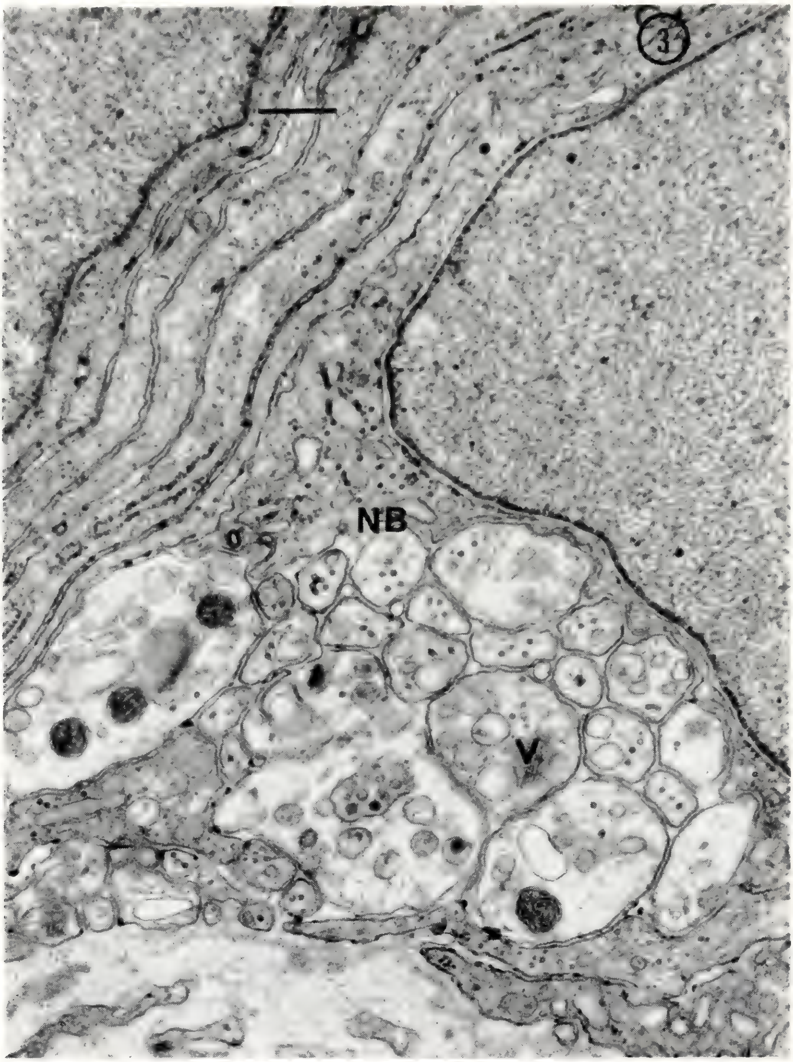


FIGURE 3. An electron micrograph of the ciliated lateral cells. A nerve bundle (NB) is visible under a lateral cell. The nerve bundle is seen to contain vesicles (V); scale = 0.2 μ.

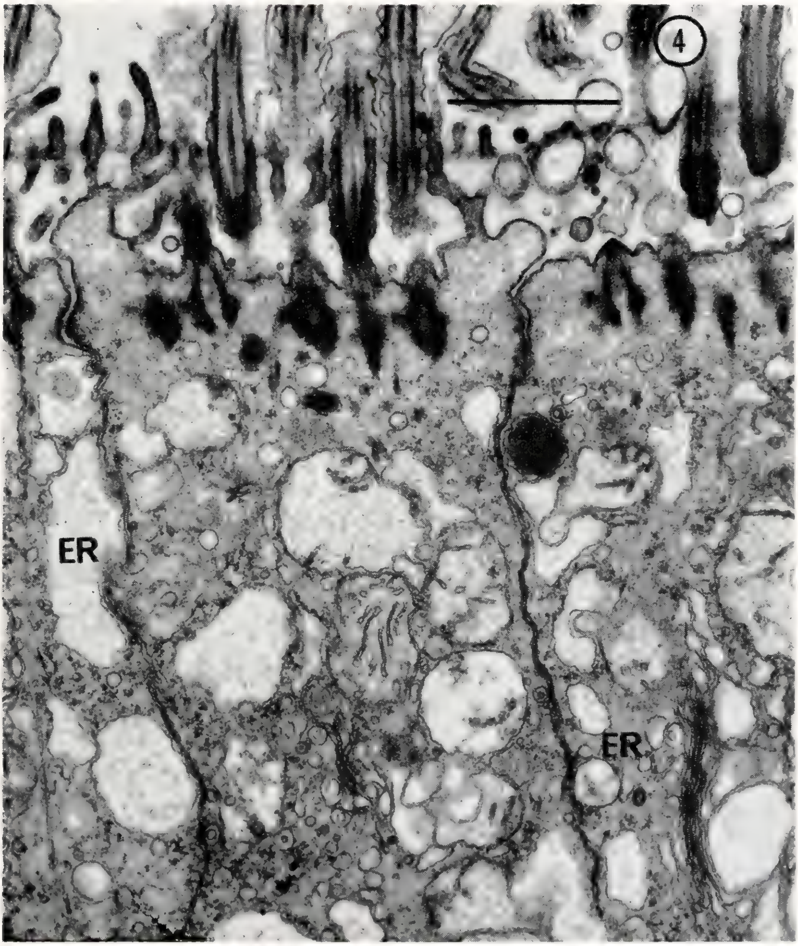


FIGURE 4. Transverse section of the gill epithelium in the lateral cell region. Note close apposition of cisternae of the smooth endoplasmic reticulum (ER) to the lateral border of the cell; scale = 1.0 μ.

associated with a flattened cisterna of endoplasmic reticulum. The lateral cells show no membrane specialization related to the proximity to the nerve fiber.

The lateral cells form an epithelium three or four cells in width. At their free surface cilia are spaced in rows separated by rows of microvilli (Fig. 4) and junctional complexes are found (Fig. 5). There are a number of large mitochondria distributed throughout the cytoplasm of these cells. There is often smooth surfaced endoplasmic reticulum running parallel to the septate junction. This junction is 0.5 μ long, and may contain as many as thirty septa. The width of a septum measures about 117 Å and the intercellular space in this region is 160 Å. In most lateral cells these septa stop abruptly and the apposing cell borders continue without interdigitation to the basal lamina. In some areas electron dense material intervenes between adjacent cell membranes (Fig. 5). Above the septate

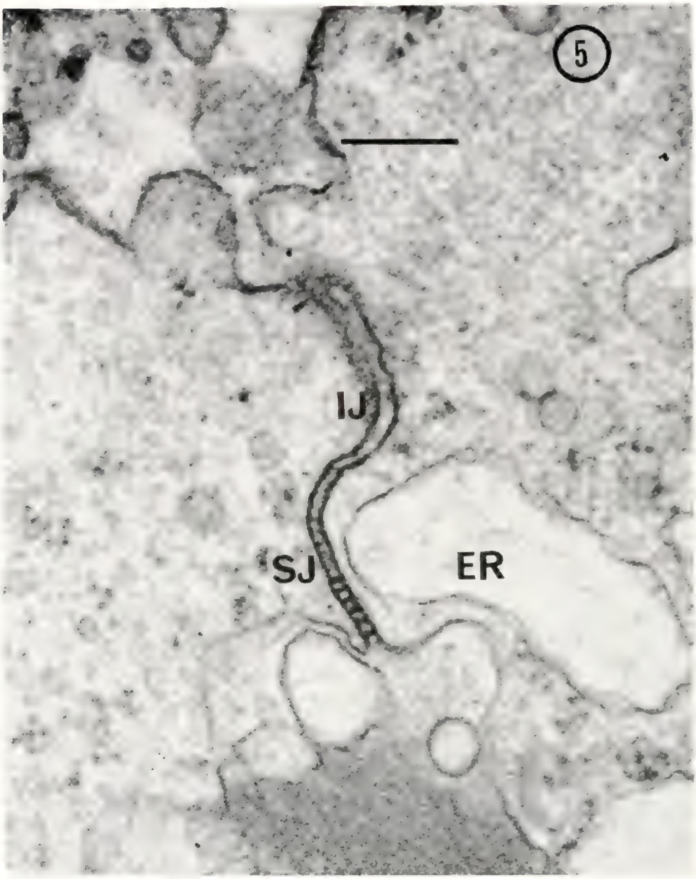


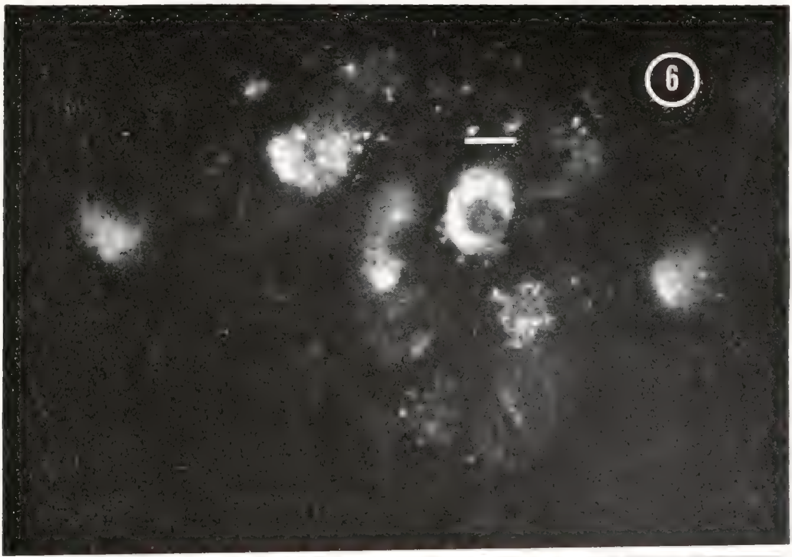
FIGURE 5. This border between cells consists of three regions: (1) an intermediary junction (IJ), (2) a septate junction (SJ) and (3) an unspecialized continuation of the cell border to the basal lamina with area of electron dense material intervening between adjacent cell membranes. Cisternae of the smooth endoplasmic reticulum lie on the side of the cell border (ER); scale = 0.2 μ.

junction the cell boundary consists of a thickened cytoplasmic surface called the intermediary junction. This region is 0.2 μ in length and in glutaraldehyde-osmium fixed material contains a dense region of amorphous material on the cytoplasmic side.

Nerve fibers reach the frontal cells as branches of nerves passing under the gill epithelium and also through the blood space within the gill filament.

Fluorescence microscopy

Formaldehyde treatment developed a yellow fluorescence characteristic of serotonin in a large number of nerve cells (Fig. 6) and axons of the branchial nerve (Fig. 7). These cells were distributed throughout the nerve, often lying close together in groups consisting of up to fifteen cells. The axons originating from the yellow cells could often be followed for a distance and consistently showed a weaker fluorescence than the cell body. In some nerve cell groups the axons left the cell mass in a dense bundle. After a short distance the fasicle separated into individual axons which then assumed an intense fluorescence as they mingled with the more spaciously distributed axons of the branchial nerve. This nerve contained strongly fluorescent yellow axons, which were smooth except in the vicinity of the gill epithelium where some of the axons exhibited varicosities. These vari-



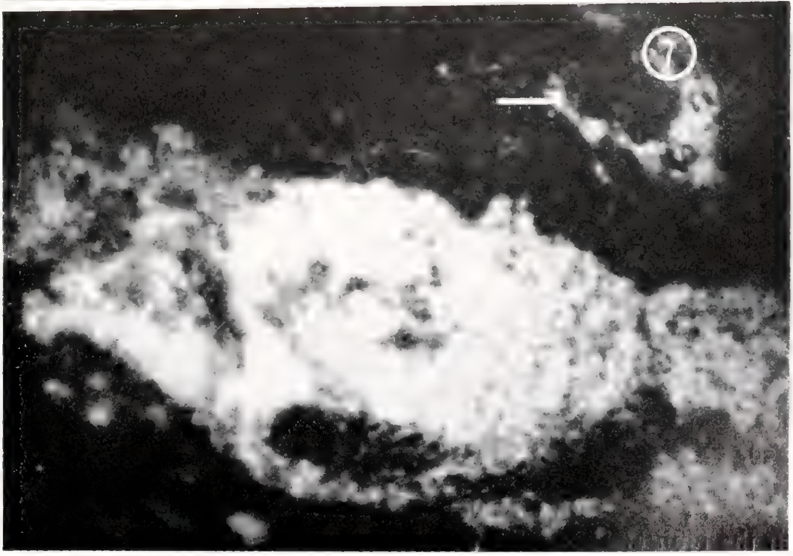


FIGURE 6. A group of serotonin-containing nerve cells; scale = 10.0 μ.
FIGURE 7. Densely packed serotonergic axons in the branchial nerve; scale = 10.0 μ.

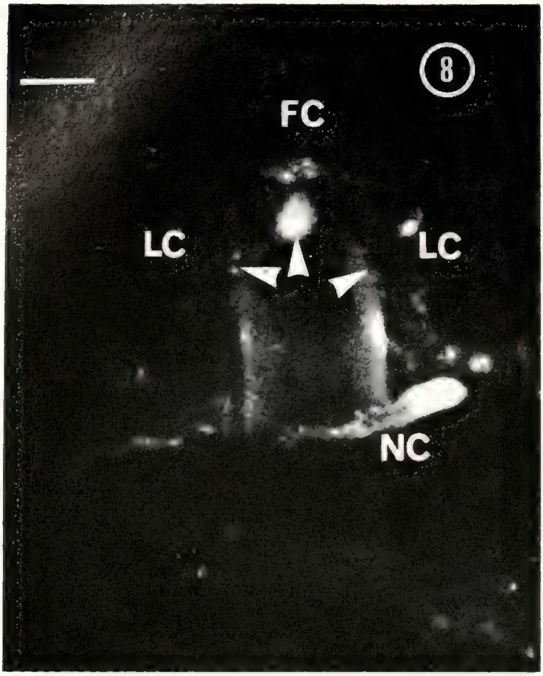


FIGURE 8. Transverse section taken through the gill showing frontal cells (FC) and lateral cells (LC) Arrows indicate specific fluorescence in the axons of the branchial nerve located under the gill epithelium. A nerve cell (NC) is seen adjacent to a gill filament; scale = 10.0 μ.

cose axons were distributed to the lateral and frontal epithelium of the gill filament. The quantity of fluorescence and number of varicosities was greatest under the frontal epithelium. No nerve cell bodies penetrated the gill epithelium but the cells were often observed adjacent to the gill filament (Fig. 8).

A few nerve cells which did not show any specific fluorescence contained coarse granules showing a yellow-orange autofluorescence. Such autofluorescent granules were also located in the branchial nerve but could readily be distinguished from the specific fluorescence.

Specimens were subjected to unilateral sectioning of the branchial nerve in the vicinity of the visceral ganglion. The gills on the intact and severed sides were excised at twenty-four hour intervals. In the distal portion of the severed nerve most of the yellow color of the nerve cells and axons had disappeared within seven days after the operation. The fluorescent material observed in nerve cells and axons situated proximal to the lesion (*i.e.*, nearest the visceral ganglion) appeared unchanged, or a considerable accumulation of fluorescence was observed. In a few cases there was increased fluorescence in the nerve cell bodies in the cortex of the visceral ganglion. The degree of fluorescence appeared unchanged in gills in which the branchial nerve was intact.

Intramuscular injection of reserpine caused a disappearance of specific fluorescence in a minimum of three days after administration.

Intramuscular injection of nialamide produced a distinct increase in yellow fluorescence in cells and axons in gills excised 24–36 hours after injection. In some cases the enhancement of fluorescence made visible the thin parts of axons between varicosities.

Discussion

There are published accounts of nervous control of cilia in various groups of molluscs. Copeland (1919, 1922) showed that ciliary activity on the foot of certain snails was under nervous control. Merton (1923) found cilio-excitatory nerves in the snail lip. Cater (1926, 1928) found a cilioinhibitory nerve in the velum of the nudibranch veliger larva. Freidenfelt (1897) postulated that the osphradium and the root of the branchial nerve constituted an independent sensory-motor center to smooth muscles in the subfilamentous structure of *Anodonta* gill. Yonge (1947) observed the sensory osphradium, lying on the base of each ctenidium in the path of the inhalant current, could sample the sediment content and possibly the chemical composition of entering current. In addition, particle transport may also be influenced by mechanical factors, and one of these, interfilamentar spacing, was reported to be under nervous control in *Ostrea virginica* (Jørgensen, 1955). Babak (1913) observed spontaneous initiation and cessation of beating of lateral cilia in various bivalves and suggested that the branchial nerve might be controlling the ciliary activity. More recently, Nelson (1951, 1960) pointed out that under normal conditions lateral cilia of *Ostrea edulis* stop beating periodically; after narcotization or isolation of these cells their cilia beat continuously. He concluded that an inhibitory neural control of lateral cilia is normally present.

There is physiological evidence that each of the ciliated cell systems in bivalve molluscs may be individually controlled (Aiello, 1960; Setna, 1940; Spittstösser, 1913; Sweeney, 1968). Aiello (1960) found that ciliary activity of the lateral ciliated cells depends on the branchial nerve, and to a lesser extent, on the cerebro-visceral connective. Furthermore, stimulation of the branchial nerve can cause both cilio-excitation and cilio-inhibition (Aiello, 1960; Paparo, 1969; Paparo and Aiello, 1970; Segerhal, 1922; Taskashi and Murakami, 1968).

In the present study branchial nerve fibers have been traced from the visceral ganglion to the ciliated epithelium of the gill, providing the morphological substratum for neural control of this epithelium in *Mytilus edulis*.

Aiello and Guideri (1965) observed that some nerve fibers enter gill filaments and course beneath the lateral ciliated epithelium in *Mytilus*. This agrees with the present observations that nerve fibers lie adjacent to gill filaments. In addition, it has been demonstrated that these nerves penetrate the fibrous basal lamina under the gill epithelium and that nerve fibers lie subjacent to postlateral and lateral ciliated cells. The nerve fibers under the ciliated lateral cells contain a polymorphic population of vesicles.

This is the first time that septate junctions have been demonstrated in the gill of *Mytilus*. They have been found connecting homologous ciliated lateral cells. Satir and Gilula (1970) have observed similar structures throughout the gill epithelium of the freshwater mussel, *Elliptio*. The number of septa observed within a junction in *Elliptio* was three times as many and the length of the junction varied between four to six times that in *Mytilus*. In numerous observations the author has consistently found a nerve fiber under only one of a series of ciliated lateral cells of a gill filament. The paucity of nerve fiber profiles close to ciliated lateral cells would support the conclusion that these cells may be excited indirecty, *i.e.*, by passive spread of current from ciliated lateral cells which are more favorably placed in relation to a nerve fiber. The first cell might act as a pacemaker in the series, conducting a stimulus to the neighboring cells, possibly *via* the septate junctions.

This is the first time that 5-HT has been specifically localized within the branchial nerve. It appears that 5-HT is released from the nerve terminals, as suggested by Aiello (1960) and not synthesized and utilized in non-neuronal ciliated cells in general. It has long been known that the branchial nerve results in a cessation of ciliary beating of the gill epithelial cells, with the frontal cells continuing to beat long after the other cells have ceased (Gray, 1929). The phenomenon may be accounted for by the greater amount of 5-HT present under the frontal epithelium as observed in this study.

The cilio-excitatory effects of 5-HT has been observed in *Mytilus* (Aiello and Guideri, 1965). Its presence has been demonstrated by photofluorometry, bioassay and paper chromatography (Gosselin, Moore and Milton, 1962; Aiello, 1962). Enzymes have been proved to exist not only for the biosynthesis of 5-HT, but also for its oxidation (Aiello, 1965; Blaschko and Milton, 1960). The evidence thus far indicates that 5-HT is the principal endogenous cilio-excitatory substance involved in the nervous control of ciliary activity in the gill of *Mytilus*.

It is well known that the ciliated lateral cells produce the water currents, that the ciilated laterofrontal cells trap particulate matter and move it onto the frontal surface, and that the ciliated frontal cells sort and transport these particles (Atkins, 1937; Dral, 1967; Orton, 1912; Wallengren, 1905). One possible advantage of independent control of the ciliated lateral cells would be the control of the flow of water over some filaments and thereby particulate matter transport over the gill.

This work was supported by USPHS grants NB05539 and GM00895. The author is indebted to Dr. Leonard L. Ross for his invaluable advice and assistance.

SUMMARY

With the use of standard dissection, electron microscopic and histochemical fluorescence techniques branchial nerve fibers have been traced from the visceral ganglion to the ciliated epithelium of the gill in the mussel, *Mytilus edulis*. Hitherto undescribed nerve fibers have been shown to lie adjacent to gill filaments and to penetrate the fibrous basal lamina under the gill epithelium. Nerve fibers have been observed subjacent to postlateral, ciliated lateral and frontal cells. Homologous lateral ciliated cells have been demonstrated to be connected by septate junctions.

The localization of 5-HT in the branchial nerve within the gill epithelium has been studied by means of histochemical fluorescence. Severing the branchial nerve or intramuscular injections of reserpine depleted the 5-HT in the gill. Intramuscular injections of nialamide resulted in an increase of specific fluorescence.

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INACTIVATION OF α- AND β-CHYMOTRYPSIN BY INTACT *HYMENOLEPIS DIMINUTA* (CESTODA)[1]

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Tapeworms, lacking a digestive tract, represent advantageous material for study of physical and chemical interactions of the host-parasite interface. Recent studies have shown that tapeworms may modify the action of host digestive enzymes, specifically those of pancreatic origin (Taylor and Thomas, 1968; Reichenbach-Klinke and Reichenbach-Klinke, 1970; Read, 1972; Pappas and Read, 1972; Ruff and Read, unpublished).

The inactivation of trypsin by the fish tapeworm *Proteocephalus longicollis* (Reichenbach-Klinke and Reichenbach-Klinke, 1970) and the rat tapeworm *Hymenolepis diminuta* (Pappas and Read, 1972) suggested that these intestinal parasites have specific protease inhibitors which protect the intact worm from host digestive enzymes. Such "anti-enzyme" protection was postulated many years ago but has largely been discredited in recent years (von Brand, 1966). The present work was undertaken to determine the possible effects of intact *H. diminuta* on α- and β-chymotrypsin.

MATERIALS AND METHODS

Specimens of *H. diminuta* from 11 day old, 30 worm infections of young male Sprague-Dawley rats (Holtzman Co.) were used in all experiments. After removal from the host, and thorough washing in 3 changes of Krebs-Ringer solution containing 25 mM Tris (hydroxymethyl) aminomethane-maleate buffer (pH 7.2) (KRT of Read, Rothman, and Simmons, 1963), worms were randomized into groups (usually 10 worms/group) and incubated in fresh KRT at 37° C for 15 min prior to their addition to the assay medium.

The assay media contained 5 ml of KRT, maintained at 37° C in a shaking water bath, plus either α- or β-chymotrypsin (45 BTEE units/mg and 28 BTEE units/mg, respectively; Sigma Chemical Co.). Groups of worms were added to the media and incubated for a predetermined time period (15 min unless otherwise noted) and removed. This period in the KRT-enzyme solution is hereafter referred to as the pre-incubation period. After removal of the worms the active enzyme was assayed by the addition of 1 ml of a prewarmed (37° C) azoalbumin (bovine origin; Sigma) solution, and the mixture incubated for 30 min at 37° C. The assay reaction was terminated by adding 1 ml of 25% (w/v) trichloroacetic acid (TCA), and the color of the TCA-soluble products measured at 420 nm following centrifugation. Some assays were also conducted using denatured bovine hemoglobin and

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[1] This study was supported by a grant (AI-01384) from the National Institutes of Health, U. S. Public Health Service.

[2] U. S. Public Health Service Postdoctoral Fellow, 5-F02-AI-45108-02.

Hammersten casein (Sigma and Mann Research Chemicals, respectively) as substrates using the methods of Bergmeyer (1963).

Assays, using azoalbumin as a substrate, were also conducted in which worms were pre-incubated in the KRT-enzyme solution for 15 min, and not removed before the addition of substrate (*i.e.,* worms were in the assay medium for the 15 min pre-incubation and 30 min assay periods). Previous control experiments (Pappas and Read, 1972) have demonstrated that *H. diminuta* does not absorb or adsorb measurable amounts of the TCA-solution products of azoalbumin digestion.

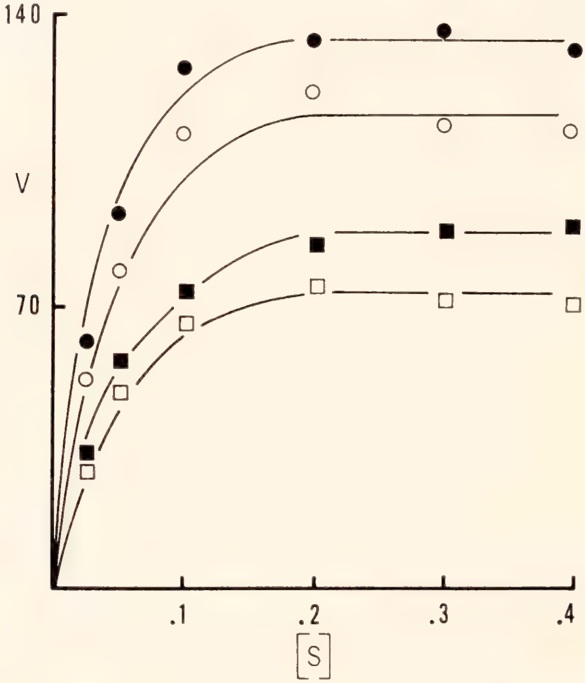

FIGURE 1. A plot of velocity (V, color change/30 min assay) of azoalbumin hydrolysis *versus* substrate concentration ([S], % azoalbumin) by α-chymotrypsin with (open circles) and without (solid circles) a 15 min pre-incubation with *Hymenolepis diminuta* and β-chymotrypsin with (open squares) and without (solid squares) a 15 min pre-incubation with *Hymenolepis diminuta.* Lines were fitted by inspection.

Experiments were conducted in which worms were incubated in KRT for 15 min, and removed, followed by the addition of either α- or β-chymotrypsin and substrate to this same KRT. These assays demonstrated that neither enzyme was affected by excretory/secretory products of the worms. Worms have also been shown previously to lack intrinsic proteolytic activity (Pappas and Read, 1972).

Experiments were performed to determine whether *H. diminuta* inactivates α-chymotrypsinogen A (Sigma Type II, from bovine pancreas; 0.15 BTEE units/mg prior to activation, 56 BTEE units/mg following activation with trypsin). Preliminary experiments were run to insure that the α-chymotrypsinogen A was inactive against azoalbumin, and that pre-incubation with *H. diminuta* had no activating effect on this zymogen. These experiments were conducted as above except that α-chymotrypsinogen A was substituted for the active enzyme preparation in assays with and without pre-incubation with worms. Subsequent experiments were as follows: Groups of *H. diminuta* were pre-incubated in 5 ml of KRT containing α-chymotrypsinogen A for 15 min and removed. Trypsin (Sigma Type XI; DCC treated to remove chymotryptic activity, 8000 BAEE units/mg) was then added to these and control media (no pre-incubation with worms); control and experimental assays were allowed to incubate for an additional 15 min at 37° C to allow for activation of the α-chymotrypsinogen A. Substrate was then added to all assays and proteolytic activity measured as described above. (*H. diminuta* has been shown not to excrete or secrete any anti-tryptic factors into KRT (Pappas and Read, 1972); therefore, the trypsin used as an activator would be expected to maintain full proteolytic activity.) Concurrent with the above experiments, assays were conducted to measure the proteolytic activity of the trypsin used to activate the α-chymotrypsinogen A so that this could be used as a correction factor at the end of the experiment.

TABLE I

Rates of hydrolysis of azoalbumin (0.3%), Hammersten casein (0.5%), and denatured hemoglobin (1.0%) (measured for a 30 min assay period) by α- and β-chymotrypsin with and without (control) a 15 min pre-incubation with Hymenolepis diminuta. *Values listed as the mean (± S.E.) of 3 replicates*

	α-Chymotrypsin			β-Chymotrypsin		
	Azoalbumin	Casein	Hemoglobin	Azoalbumin	Casein	Hemoglobin
Control	134 ± 1	710 ± 8*	135 ± 3*	81 ± 1	377 ± 4*	91 ± 2*
Experimental	115 ± 2	632 ± 6	119 ± 2	64 ± 1	300 ± 4	76 ± 1
Per cent inactivation	15	11	12	21	20	17

* Rates for both casein and denatured hemoglobin were determined according to the method of Bergmeyer (1963) for that of hemoglobin.

Unless otherwise noted, experiments were conducted under the following conditions: Enzyme concentration ([E], α- or β-chymotrypsin) = 20 μg/assay; substrate concentration ([S], azoalbumin) = 0.3%. In addition, all points represented in the figures are the means of 3 replicates, and lines were fitted by regression analysis. The regression equations have been omitted since they are not important in interpreting the results. Without exception, the correlation coefficients of lines fitted by regression analyses were $\geqslant 0.99$ (or $\geqslant -0.99$).

RESULTS

Both α- and β-chymotrypsin were inactivated when pre-incubated with intact *H. diminuta.* The inactivation was not related to the substrate used in assaying chymotryptic activity (Table I). When the substrate concentration was varied at a constant enzyme concentration, both enzymes exhibited typical Michaelis-Menten kinetics (Fig. 1). Further, partially inactivated α- and β-chymotrypsin exhibited kinetics similar to untreated enzymes (Fig. 1) indicating that the enzymes exposed to *H. diminuta* had indeed been rendered catalytically inactive rather than undergoing some kind of decreased substrate affinity. To insure that the observed inactivation was not due to the removal of some enzyme upon removal of the worms from the assay medium, assays were conducted in which the worms were allowed to remain in the assay medium for the entire assay period (45 min). With both α- and β-chymotrypsin, inactivation was greater than in assays where worms were only pre-incubated for 15 min in the presence of enzyme (Fig. 1). This indicated that the apparent inactivation was not simply due to removal of the enzyme with worms.

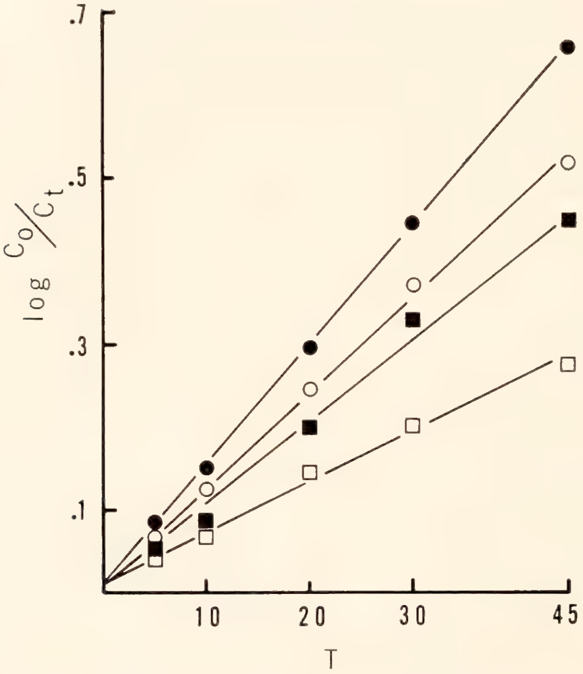

FIGURE 2. A plot of log (C_o/C_t) (where: C_o = concentration at time O, and C_t = concentration at time T) *versus* time (T, min) for the hydrolysis of azoalbumin by α- and β-chymotrypsin with and without a 15 min pre-incubation with *Hymenolepis diminuta*. All symbols as in Figure 1.

When native and partially inactivated enzymes were assayed as a function of time, hydrolysis was first order (Fig. 2); these data also showed that inactivation of both chymotrypsins was not reversible, at least within the 45 min period tested. That is, the per cent inactivation of α- and β-chymotrypsin remained constant for up to 45 min after removal of the worms.

Various concentrations of α- and β-chymotrypsin were incubated with *H. diminuta* and subsequently assayed after removal of the worms. Hydrolysis rates were not directly proportional to the enzyme concentrations (Fig. 3). This would be expected since the reactions showed first order kinetics at this substrate concentration (Fig. 2). The data of Figure 3 indicated that the amount of enzyme inactivated was a function of enzyme concentration only at very low concentrations; *i.e.*, at high enzyme concentrations, the absolute amount of enzyme inactivated by *H. diminuta* was constant regardless of the relative amount of enzyme present.

At enzyme concentrations of 20 μg/assay, the inactivation of α- and β-chymotrypsin was a linear function of the time of exposure of the enzymes to *H. diminuta* (Fig. 4). In this experiment worms were incubated with one of the chymotrypsins for varying time periods and removed, and the medium assayed for protease activity using a fixed time period assay. At this enzyme/worm ratio, inactivation showed zero order kinetics.

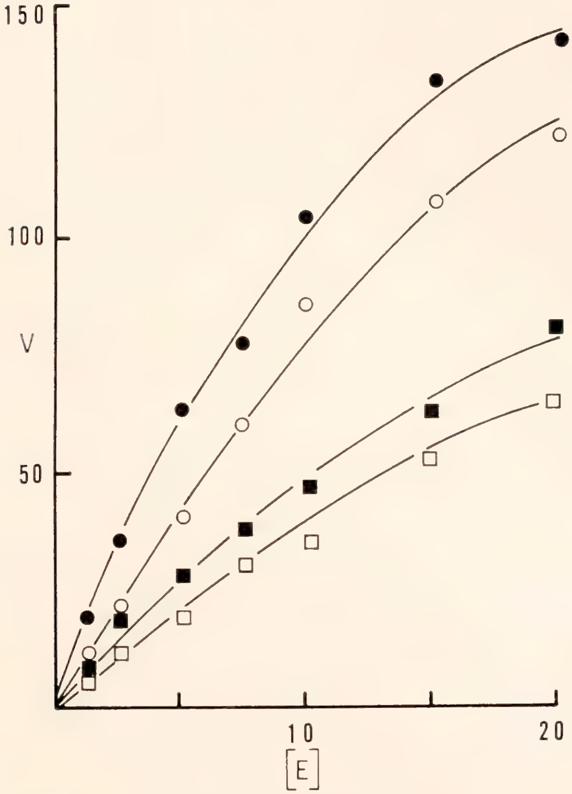

FIGURE 3. A plot of velocity (V, color change/30 min assay) of azoalbumin hydrolysis *versus* enzyme concentration ([E], μg/6 ml assay) by α- and β-chymotrypsin with and without a 15 min pre-incubation with *Hymenolepis diminuta*. All symbols as in Figure 1.

When worms were incubated successively in 3 fresh solutions of α- or β-chymotrypsin, all incubations for 15 min, subsequent assays of enzyme activity revealed equal enzyme inactivation in each successive solution (Table II). Clearly, exposure to the chymotrypsins does not alter the worms' capacity to inactivate these enzymes.

Table II

Rates (color change/30 min assay) of azoalbumin hydrolysis by α- and β-chymotrypsin after pre-incubation with Hymenolepis diminuta. *Worms were pre-incubated in the first set (#1) of assays for 15 min, and successively transferred to the second (#2) and third (#3) sets for 15 min each. Assays were conducted following removal of the worms. Control assays (no pre-incubation with worms) were conducted to insure the stability of the enzyme solutions.*

	α-Chymotrypsin		β-Chymotrypsin	
	Controls	Experimentals	Controls	Experimentals
Set #1	133.50 ± 0.96*	105.50 ± 1.94*	80.66 ± 1.91**	64.33 ± 1.01**
Set #2	129.75 ± 2.01	106.50 ± 1.50	82.33 ± 1.09	66.00 ± 1.29
Set #3	133.00 ± 1.29	108.25 ± 1.44	83.00 ± 1.36	65.00 ± 1.99

* Values reported as mean of 4 replicates ± S.E. Two-way analysis of variance of replicate data yielded the following: $F_{[2,18]}$ (rows) = 1.28, $P > 0.25$; $F_{[1,18]}$ (columns) = 392.70, $P \ll 0.001$; $F_{[2,18]}$ (interaction) = 1.20, $P > 0.25$.

** Values reported as mean of 3 replicates ± S.E. Two-way analysis of variance of replicate data yielded the following: $F_{[2,12]}$ (rows) = 2.26, $P > 0.10$; $F_{[1,12]}$ (columns) = 123.98, $P \ll 0.001$; $F_{[2,12]}$ (interaction) = 0.06, $P > 0.75$.

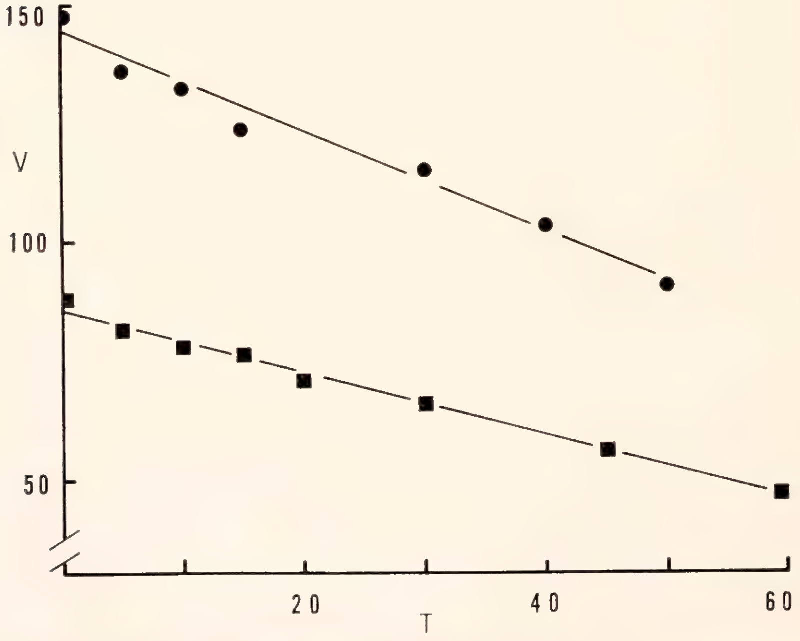

Figure 4. A plot of velocity (V, color change/30 min assay) of azoalbumin hydrolysis *versus* length of time (T, min) that *Hymenolepis diminuta* was pre-incubated in α- and β-chymotrypsin (solid circles, and solid squares, respectively).

Inactivation of α- and β-chymotrypsin was a function of the number of *H. diminuta* to which the enzymes were exposed; in the case of β-chymotrypsin, this relationship was linear up to 20 worms/assay (Fig. 5). To determine whether inactivation was a function of worm surface area rather than of worm weight, experiments were conducted using worms of identical age but varying in size (see Pappas and Read, 1972, for methods). The data (Table III) indicated that inactivation of α- and β-chymotrypsin was not a function of available surface area, but a function of total tissue weight (Fig. 4).

If surface adsorption were involved in inactivating the chymotrypsins polyions might be expected to block or interfere with inactivation. Therefore, worms were incubated with poly-L-arginine (M.W. *ca.* 65,000), poly-L-aspartic acid (M.W. *ca.* 27,000), heparin (170 units/mg) or purified yeast RNA (all at 10 μg/ml and obtained from Sigma) for 15 min, followed by incubation with α- or β-chymotrypsin in the presence of these same polyions. Subsequent assays, after removal of the worms, showed that inactivation of the chymotrypsins was unaffected by the addition of polyions. Control assays demonstrated that none of the above listed polyions affected either enzyme in the absence of worms.

TABLE III

Effect of worm number, with constant worm weight, on the inactivation of α- and β-chymotrypsin by Hymenolepis diminuta. *Worms were obtained, 15 days post-infection, from rats which had been infected initially with different numbers of cysticercoids (left-hand column). Values reported as the mean (± S.E.) of 4 replicates*

			Rate of hydrolysis*	
Number of cysticercoids per original infection	Number of worms/assay	Weight range**	α-chymotrypsin	β-chymotrypsin
10	2	909–925	116.50 ± 0.50	87.75 ± 1.11
30	6	878–925	111.25 ± 1.44	92.25 ± 1.11
50	9	907–932	116.00 ± 4.06	93.50 ± 2.18

* Measured as color change/30 min assay. Rates for control assays (no pre-incubation with worms) were as follows: α-chymotrypsin = 146.25 ± 2.85; β-chymotrypsin = 103.75 ± 0.48. One-way analysis of variance of replicate data yielded the following: α-chymotrypsin data, $F_{[2,9]} = 1.34$, $P > 0.25$; β-chymotrypsin data, $F_{[2,9]} = 3.80$, $P > 0.05$.

** mg wet weight of total worms in assay, listed as the weight range of the 4 assay groups.

It seemed desirable to determine whether the worms were capable of inactivating α-chymotrypsinogen A, a precursor of the chymotrypsins. Such an experiment is shown in Table IV. As indicated, worms were present only during the pre-incubation period. In C and D of Table IV, trypsin was added to activate the α-chymotrypsinogen A, and these values must therefore be corrected for this added activity by subtracting E. Thus, C minus E of Table IV gives the value for the chymotryptic activity (130 units), and D minus E gives the chymotryptic activity after exposure of the chymotrypsinogen to *H. diminuta* (81 units). Therefore, pre-incubation with *H. diminuta* resulted in a 38% decrease in the amount of α-chymotrypsinogen A activated. Appropriate controls demonstrated that worms

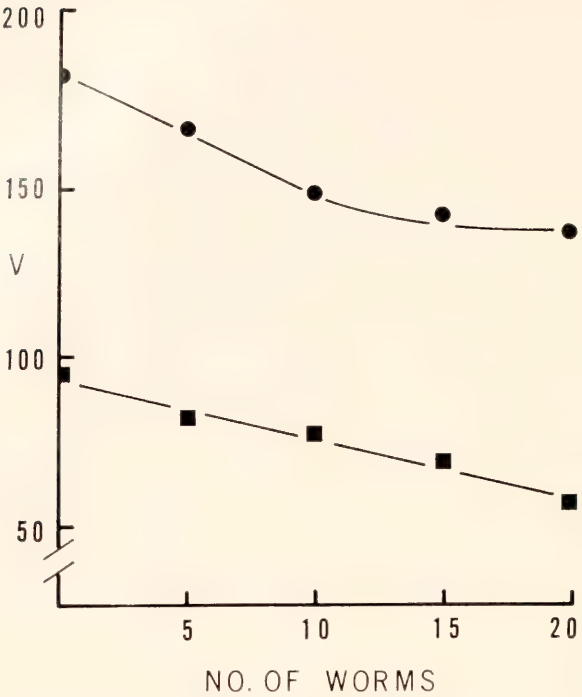

FIGURE 5. A plot of velocity (V, color change/30 min assay) of azoalbumin hydrolysis *versus* number of *Hymenolepis diminuta* pre-incubated with α- and β-chymotrypsin. Symbols as in Figure 4. The line for α-chymotrypsin was fitted by inspection while that for β-chymotrypsin was fitted by regression analysis.

TABLE IV

Results of an experiment to determine whether Hymenolepis diminuta *inactivates α-chymotrypsinogen A, The experiment consisted of 6 different assays (A–F), each beginning with the ingredients listed in the left-hand column. After a pre-incubation of 15 min, the procedures listed in the middle column were performed, and substrate added*

Pre-incubation components	Incubation additions or deletions	Rate*
(A) α-chymotrypsinogen A**	None	1.67 ± 0.67
(B) α-chymotrypsinogen A + worms	Worms removed	0
(C) α-chymotrypsinogen A	Trypsin added**†	184.67 ± 1.67
(D) α-chymotrypsinogen A + worms	Worms removed, trypsin added**†	135.00 ± 2.65
(E) Trypsin**	None	54.67 ± 1.20
(F) Trypsin + worms	Worms removed	46.00 ± 0.57

* Color change/30 min assay. Means of 3 replicates ± S.E.

** Final concentration = 20 μg/assay.

† In these assays, the trypsin + α-chymotrypsinogen A solution was allowed to incubate for 15 min at 37° C for activation of the zymogen.

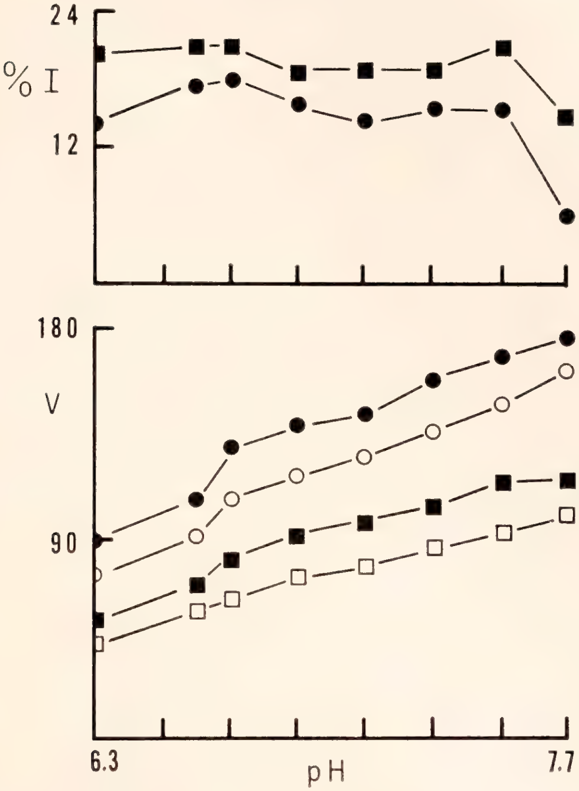

FIGURE 6. Plots of velocity (V, color change/30 min assay) of azoalbumin hydrolysis (lower graph) and per cent inactivation (%I, upper graph) *versus* pH of the incubation medium (0.2 pH unit increments) for α- and β-chymotrypsin with and without a 15 min pre-incubation with *Hymenolepis diminuta.* Symbols in lower and upper graphs as in Figures 1 and 4, respectively. Lines were fitted by inspection.

did not activate the inactive zymogen (A and B, Table IV). It may be noted that E minus F of Table IV shows that trypsin itself is inactivated by the intact worm as reported by Pappas and Read (1972).

When the effect of pH on the inactivation of α- and β-chymotrypsin was examined, the results were as shown in Figure 6. Between pH 6.3 and 7.5, pH clearly had a negligible effect on enzyme inactivation. There is a suggestion that there may be decreased inactivation at pH 7.7.

DISCUSSION

Some of the results of this study resemble the findings of our earlier study dealing with the effects of *H. diminuta* on pancreatic trypsin (Pappas and Read. 1972). That is, inactivation of α- and β-chymotrypsin is dependent on the period of exposure of the worms to the enzyme; inactivation appears to be irreversible; inactivation is not dependent upon the nature of the substrate nor is it blocked by polyions which might be expected to affect electrostatic charges associated with the surface of *H. diminuta* (Lumsden, 1972). Further, inactivation does not appear to be a function of worm surface area, but rather is a function of worm weight. In all of these respects, the inactivation of the chymotrypsins resembles the effects of *H. diminuta* on trypsin.

Pappas and Read (1972) postulated that trypsin inactivation by *H. diminuta* involves the interaction of this enzyme with an inhibitor associated with the glycocalyx of this parasite. It was suggested that this inhibitor is produced continually at a rate which is a function of worm weight rather than surface area. That the glycocalyx of *H. diminuta* is continually replaced, in as short a period as 6 hr, has been shown by Oaks and Lumsden (1971), and Pappas and Read (1972) suggested that glycocalyx turnover may be more approximately a function of tissue weight than of surface area. Therefore, it is suggested that a mechanism similar to that of trypsin inactivation is also involved in the inactivation of chymotrypsins.

Simple binding of α- or β-chymotrypsin to the worm surface seems to be ruled out on several grounds: Inactivation follows zero order kinetics for periods up to 60 min; inactivation does not appear to be surface area dependent; previous exposure to the chymotrypsins does not affect the rate of chymotrypsin inactivation when the worms are exposed to fresh enzyme; and polyions are without effect on chymotrypsin inactivation.

Although the inactivation of trypsin and the chymotrypsins seem similar, there is a suggestion that the processes are separate. While the inactivation of trypsin shows a definite pH optimum at a slightly basic pH (Pappas and Read, 1972), optimal inactivation of the chymotrypsins was observed to occur over a broad pH range (6.3–7.5). This requires further study.

It is of interest that the inactivation of β-chymotrypsin is a linear function of worm number up to 20 worms/assay, whereas the inactivation of α-chymotrypsin is not. While it may be argued that this suggests separate mechanisms for the inactivation of the chymotrypsins, the pH data do not substantiate this. Further experiments will be necessary to clarify this question.

The formation of α-chymotrypsin *in vivo* involves the splitting of specific peptide and disulfide linkages of the inactive zymogen, α-chymotrypsinogen A, with the formation of π- and δ-chymotrypsin as intermediate products. In turn, β- and γ-chymotrypsin can be formed from α-chymotrypsin (Desnuelle, 1960). Since we have shown that α-chymotrypsinogen A and α- and β-chymotrypsin are inactivated in the presence of intact *H. diminuta*, we may speculate that the worm is probably capable of inactivating π-, δ-, and γ-chymotrypsin as well, since these are products of α-chymotrypsinogen A and/or α-chymotrypsin.

Although the view that parasites of the gut may utilize anti-enzymes as a mechanism for somatic protection from the digestive enzymes of the host has not been widely accepted, the present study and those of Reichenbach-Klinke and Reichenbach-Klinke (1970) and Pappas and Read (1972) suggest that the role of anti-enzymes in the ecology of intestinal parasites may require further consideration. Trypsin is inactivated by *H. diminuta*, the rat tapeworm, and tryptic activity is inhibited by intact *Proteocephalus longicollis*, a tapeworm of fishes (Reichenbach-Klinke and Reichenbach-Klinke, 1970). Although the latter authors assumed that a trypsin inhibitor was secreted into the surrounding medium by the tapeworms, no experimental evidence for such a secretion was furnished. In the present study, and in the study of Pappas and Read (1972), it has been shown that there is no secretion of trypsin or chymotrypsin inhibitors into the medium by *H. diminuta.*

It is of interest to compare the interaction of *H. diminuta* with the chymotrypsins to the known interactions of this worm with other digestive enzymes. As noted above, inactivation of trypsin and α- and β-chymotrypsin seem to occur by similar mechanisms. However, pancreatic lipase is inhibited rather than inactivated by *H. diminuta,* and the inhibition of lipase is readily reversible (Ruff and Read, unpublished). Further, the inhibition of lipase is a function of available surface area. Pancreatic α-amylase activity is enhanced in the presence of *H. diminuta* (Read, 1972). The effects of the worm on lipase and α-amylase activity have been explained as an adsorption phenomenon, the adsorption resulting in stabilization of the enzyme in an unfavorable (lipase) or favorable (α-amylase) configuration for catalytic activity (Read, 1972; Ruff and Read, unpublished).

Membrane-bound phosphohydrolases have also been demonstrated in *H. diminuta* (Arme and Read, 1970; Dike and Read, 1971a, 1971b), but the intact worm exhibits no proteolytic activity with azoalbumin or casein (Pappas and Read, 1972), nor amylolytic activity (Read, 1972). There is also abundant evidence that the surface of *H. diminuta* is specialized for the absorption of a variety of low molecular weight metabolites. These facts, taken together, suggest that the external syncytial epithelium of *H. diminuta,* an important component of the host-parasite interface, serves a complicated digestive, absorptive, and protective function.

The technical assistance of Mr. William Kitzman is gratefully acknowledged.

SUMMARY

When specimens of intact *Hymenolepis diminuta* were incubated in the presence of α- or β-chymotrypsin, assays for proteolytic activity following removal of the worms showed an inactivation of both enzymes. The amount of inactivation in either case was dependent upon the enzyme concentration, total number of worms (total worm weight) present, period of time worms were incubated with the enzymes, and pH of the assay medium. Inactivation of α- and β-chymotrypsin was independent of available surface area and the presence of polyions, was irreversible, and ceased upon removal of the worms from the medium. Intact worms also inactivited the zymogen, α-chymotrypsinogen A. The data suggest that the inactivation of α- and β-chymotrypsin resembles that previously reported for inactivation of trypsin by intact *H. diminuta.*

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OBSERVATIONS ON THREE SPECIES OF JELLYFISHES FROM CHESAPEAKE BAY WITH SPECIAL REFERENCE TO THEIR TOXINS. II. *CYANEA CAPILLATA*[1]

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Cyanea capillata, the pink jellyfish or lion's mane, occurs in Chesapeake Bay and its tributary rivers from the latter part of November to the first of May. The largest specimens attain an umbrellar diameter of eight inches, but most specimens observed are in the range of four to six inches. Pigmentation varies from pink to brown in different specimens. Compared to *Chrysaora quinquecirrha* the much more numerous tentacles of *Cyanea* are vastly shorter even when fully extended. The anatomical features of the Chesapeake Bay *Cyanea* are identical in every respect to those of the North Atlantic form for which a record eight feet umbrellar width (Miner, 1950) has been reported.

Cleland and Southcott (1965) detail reports by various observers (Wood, 1874; Kristenson, 1949; Uvnäs, 1960) on the stinging ability of *C. capillata*, and other reports (Pope, 1953a, 1953b; Barnes, 1960) of stingings by *C. annaskala*. On the other hand Burnett (1971) reports that the smaller *C. capillata* of Chesapeake Bay (page 70) "rarely produce symptomatic stings in humans."

Although Mayer (1910) believes that *C. capillata* and *C. annaskala* are distinct northern and southern hemispheric species with many intergrading varieties in each, Kramp (1961), however, states that there is only one species of *Cyanea*, namely *C. capillata* which is cosmopolitan in distribution.

Halstead (1965), in a table adapted from several sources, lists three distinct types of nematocysts from *Cyanea*. Cleland and Southcott (1965), however, find two, and perhaps three, different types in *Cyanea* sp. (*C. annaskala* ?). Burnett (1971) describes two types of nematocytes (cnidoblasts) in the tentacles of the Chesapeake Bay *Cyanea*. Presumably these give rise to the mature types of nematocysts found in adults.

Rice and Powell (1970) reported observations on *Chrysaora quinquecirrha* related to (1) the extraction and isolation of nematocyst toxin; (2) toxicity experiments; and (3) the determination of the chemical nature of the toxins. The present paper presents our observations on *C. capillata* along these same lines. Reference may be made to the earlier paper for literature which might be relevant here as well.

MATERIALS AND METHODS

Jellyfishes were collected in the Piankatank River near Deltaville, Virginia during the months of January, February, March and April, 1967–1971. Because

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[1] This investigation was supported by several Faculty Research Grants of the University of Richmond and a Virginia Academy of Science Research Grant.

it was difficult and tedious to secure tentacles only, entire animals were used. Although this entailed the processing of much unwanted material, nematocysts from the oral lobes and exumbrella were obtained in addition to those on the tentacles.

Nematocyst toxin was extracted by the same method that was used for *Chrysaora* (Rice and Powell, 1970). This involved the following processes: autolysis of the material in the refrigerator; screening through nylon netting and silk bolting cloth; sedimentation; decanting the supernatant; centrifugation and washing of the nematocysts; homogenization of the nematocysts; centrifugation at 18,500 rpm; and subsequent collection of the supernatant containing the toxin.

Since a rather large amount of lipid material was encountered as a result of using whole animals, extensive centrifugation and washing was necessary to remove the lipid associated with the nematocysts.

White Swiss mice (Wistar strain) were used as test animals in all of the toxicity experiments. All materials were suspended in 0.9% NaCl solution and injected intraperitoneally in 1 ml doses. Mice injected with 0.9% NaCl solution served as controls.

The following standard reagents were used for chemical tests: Biuret, ninhydrin, Molisch, and Benedict's. Van Gieson's picrofuchsin and Mallory's aniline blue strains were applied to nematocysts.

The electrophoresis pattern of the nematocyst toxin was obtained using polyacrylamide gels. The standard separating gel of 7% acrylamide provided by Canalco Instrument Corporation was used at a pH of 9.5 according to their instructions for disc electrophoresis.

RESULTS

The stinging ability of the Chesapeake Bay *Cyanea* was tested by using mature volunteers of both sexes and various ages up to sixty years. When a living tentacle was pressed tightly with a glass slide against the biceps area of the arm no reaction was observed in 42 subjects. Six, however, thought they felt a faint stinging sensation. When faradic shock was applied to a tentacle, the 6 subjects tested were unaffected. Since jellyfish stingings usually take place in the water, and water softens the skin, experiments were performed after the biceps area of both arms was subjected to a 1.5% salt water solution for 15 minutes. One arm served as a control. Twenty subjects were unaffected; 15 subjects reported a faint tingling sensation. In only a few cases was mild erythema observed.

The tentacles of *Cyanea* are covered with almost contiguous batteries of nematocysts, each battery consisting of from 2 to 4 dozen or more nematocysts. The batteries near the base of each tentacle are only about half the size of the distal ones. Batteries also occur on the marginal portions of the oral lobes with scattered nematocysts elsewhere. There are no nematocysts on the subumbrellar surface except for some relatively small batteries on the folds surrounding the gonads. Scattered batteries occur on the exumbrellar marginal areas of the lappets. Toward the center of the bell these give way to occasional isolated individual nematocysts.

Three types of nematocysts were observed: large atrichous isorhizas; medium sized heterotrichous microbasic euryteles; and small holotrichous isorhizas. More than half of the nematocysts in a tentacle battery are euryteles and, with the exception occasionally of one or two atrichous isorhizas, the remainder are holotrichous isorhizas. The capsules of the atrichous isorhizas ranged in length from 15 to 20 μ and in width from 8 to 12 μ. The discharged tubes of these measured from 500 to 1400 μ in length. The capsules of the heterotrichous microbasic euryteles ranged in length from 8 to 13 μ and in width from 5 to 8 μ. The tubes measured from 100 to 400 μ in length. The butts were 8 to 13.0 μ in length. The capsules of the holotrichous isorhizas ranged in length from 6 to 8 μ and in width from 4 to 5 μ. The tubes measured from 50 to 200 μ in length. The tubes of all three types are of the order of magnitude of 0.5 μ in width.

The discharge of nematocysts from living tentacles was observed microscopically after the application of pressure, faradic electrical shock and chemical reagents such as formalin-acetic-alcohol (FAA). However, these were ineffective on isolated nematocysts. When an entire living jellyfish in sea water was stimulated by electrical shock, it contracted convulsively. Nematocysts on a few tentacles in line with the flow of current discharged but those on most of the tentacles did not. If isolated tentacles were stimulated, large numbers of nematocysts discharged but many were unaffected. Continued shocks in the same region had no further effect.

Homogenization of heavy suspensions of nematocysts in the frozen state was effective in rupturing 60–80% of them.

The penetration power of nematocysts was studied using Grenacher's borax-carmine agar (2%) as described in an earlier paper (Rice and Powell, 1970). When living tentacles in contact with the agar were stimulated by the application of FAA, nematocysts were discharged to various distances into the gel. Numerous measurements showed that the vast majority of tubes penetrated the agar a distance of from 200 to 350 μ, a few up to 400 μ, and an occasional one up to 1 mm. The paths of tubes through the agar were sinuous, few were straight. Many of the tubes were coiled on themselves.

The following materials were used in toxicity experiments: the supernatant after centrifugation of homogenized nematocyst suspensions at 18,500 rpm for one hour; washed undischarged capsules; washed capsular debris and tubes; and washed lipid collected from the surface of supernatant. Injections of supernatant (minus lipid) into mice were quite toxic. The LD 50, based on air dried residues corrected for salt content, was of the order of magnitude of 6 μg/g of body weight. Washed capsular debris and tubes had no observable effect on mice, even in heavy, milky suspensions. When washed undischarged nematocysts in moderately heavy suspensions were injected, the mice seemed not to be affected; heavier suspensions, however, were fatal. Lipids in heavy suspension had no apparent toxic effect.

All of these materials except the lipid were subjected to the following reagents: Biuret, ninhydrin, Benedict's, and Molisch. The washed undischarged nematocysts gave a positive Biuret test and the supernatant was Biuret and ninhydrin positive. All other tests were negative. The positive tests show that the toxin is contained in the nematocysts and that it is proteinaceous.

Electrophoresis studies of the supernatant using polyacrylamide gel and subsequently staining with Coomassie blue, confirm the protein nature of the toxin.

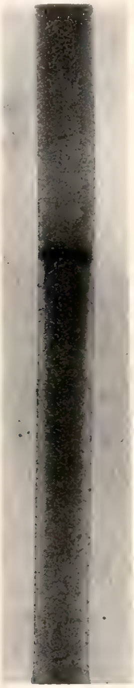

FIGURE 1. Electrophoretogram of the toxin of *Cyanea* nematocysts obtained using polyacrylamide gels with subsequent staining with Coomassie blue stain for protein; origin at the top of the figure.

Figure 1 is a photograph of a typical electrophoretogram. A single, sharply defined band indicates that the toxin is a single protein.

Whatever the chemical nature of nematocyst capsules and tubes, staining with aniline blue or picrofuchsin, accepted vertebrate collagen stains, was negative.

DISCUSSION

As noted by Burnett (1971) and as observed by us independently in experiments reported here, the Chesapeake Bay *Cyanea* is incapable of stinging most individuals. Any reaction that does occur is so mild and so short-lived as to be barely noticeable. These observations are in strong contrast to reports by others (Cleland and Southcott, 1965) who report painful experiences with Mayer's (1910) northern and southern hemispheric *Cyanea* which Kramp (1961) maintains belong to the same species.

Although the reason for the difference in stinging ability of the Chesapeake Bay *Cyanea* and that found elsewhere is not known, it hardly seems likely that it is due to its smaller size. Unpublished observations on *Chrysaora quinquecirrha* show, that for equal surface areas of skin affected, small specimens produce just as severe a reaction as do larger ones. It may be that the difference in stinging ability is due to a difference in the power of penetration of the nematocysts. Although the Chesapeake Bay *Cyanea* and those found elsewhere have the same types of nematocysts, it may be that the Chesapeake form has a much lower percentage of the type which has the power to penetrate the skin. Some evidence of a difference in penetrating powers of nematocysts of *Chrysaora* and *Cyanea* has been observed in our laboratory.

A comparison of these two species shows that: (1) nematocyst batteries of *Chrysaora* consist mostly of atrichous and holotrichous isorhizas and fewer euryteles whereas batteries of *Cyanea* are made up of euryteles and holotrichous isorhizas and few atrichous isorhizas; (2) the atrichous isorhiza tubes of *Chrysaora* have a maximum length of 2 mm compared to 1.4 mm in *Cyanea;* and (3) the paths of penetration of tubes of *Chrysaora* into agar are straight while those of *Cyanea* are sinuous and often coiled on themselves. These facts would seem to indicate that most, if not all, of the effective toxin in *Chrysaora* stings comes from the atrichous isorhizas, which may penetrate deeper into the skin and which are present in larger numbers as compared to the same type in *Cyanea*, and consequently explain why the latter rarely produces any symptoms.

Electric shock has been used successfully to effect discharge of nematocysts from living Cnidaria (Kline and Waravdekar, 1960; Barnes, 1967). We found faradic shocks just as effective in producing discharge of nematocysts in *Cyanea* as in *Chrysaora* (Rice and Powell, 1970). The same was also true of FAA fixative.

As in *Chrysaora,* both of the above agents were ineffective in causing discharge of isolated nematocysts although Phillips and Abbott (1957) were successful in obtaining by various chemical means the discharge of nematocysts of *Metridium senile fimbriatum.* Rupture or discharge of the nematocysts of *Cyanea* was not produced to any observable degree by shock or FAA treatment which is in agreement with similar results for *Chrysaora.* This contrasts with the work of Burnett, Stone, Pierce, Cargo, Layne and Sutton (1968) who had positive results with a number of physical and chemical agents. Homogenization of frozen nematocysts of *Cyanea* caused the rupture of 60 to 80%.

It has been stated earlier that the paths of the nematocyst tubes of *Cyanea* in agar were sinuous as compared to the straight paths of *Chrysaora.* No confirmed explanation for this difference has been formulated. It could be due to differences in rigidity, size, shape, or force of discharge of the tubes.

In *Cyanea*, as in *Chrysaora*, the evidence shows that: (1) the toxic principle is contained in the capsules of the nematocysts; (2) it is protein; (3) the capsules and tubes are not toxic; (4) associated lipid is not toxic; and (5) the chemical nature of the capsules and tubes is unlike that of vertebrate collagen.

The LD 50 (6 μg/g) of the *Cyanea* toxin as compared to that of *Chrysaora* (16–19 μg/g) indicates a greater toxicity of the former for mice.

We are indebted to the following persons for assistance: Mr. William A. Dorsey, Chief of Public Laboratories, Richmond City Health Department for supplying us with white mice; Dr. Wilton R. Tenney, Department of Biology, University of Richmond for the photograph of the electrophoretogram; and Dr. Francis B. Leftwich, Department of Biology, University of Richmond for technical assistance.

SUMMARY

1. The human body exhibits little or no response to contact with the tentacles of specimens of *Cyanea capillata* from Chesapeake Bay.

2. Three types of nematocysts were identified: atrichous isorhizas, holotrichous isorhizas, and heterotrichous microbasic euryteles.

3. The tubes of nematocysts discharged into 2% agar take a sinuous course, often coiling on themselves. Most tubes penetrated less than 350 μ.

4. Although faradic shock and FAA caused discharge of nematocysts from living tentacles, they failed to produce discharge of isolated nematocysts.

5. Homogenization of thoroughly washed frozen nematocysts causes rupture, releasing the toxic principle which is a single protein.

6. The LD 50 of the toxin was of the order of magnitude of 6 μg/g of mouse body weight.

7. Mice were unaffected by intraperitoneal injections of washed capsular debris and tubes, or lipids, or of washed undischarged nematocysts in moderately heavy suspensions.

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EARLY LIFE-HISTORY OF *MELAMPUS* AND THE SIGNIFICANCE OF SEMILUNAR SYNCHRONY[1]

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Living in the higher levels of salt marshes, pulmonate snails of the genus *Melampus* are truly "amphibious." In their respiration, they are land snails breathing air through pneumostome and lung. In their reproduction, they are primitive marine snails spawning large numbers of small eggs which yield planktonic veliger larvae on hatching. As adumbrated in earlier publications on the biology of *Melampus bidentatus* (Russell Hunter and Apley, 1966; Apley, Russell-Hunter and Avolizi, 1967; Apley, 1970; Russell-Hunter, Apley and Hunter, 1970), there is a significant temporal "fitting" of the reproductive events including egg-laying and hatching to the two-week periodicity of spring tides. The sequence and "control" of these events and, in addition, of those of veliger settlement, metamorphosis and spat growth have now been examined in greater detail and the more significant observations are reported in this paper. Minor parts of this account are derived from observations in the summers of 1964 and 1966, but most of the detailed observations on sequential "timing," the experiments on "controls" and the observations on laval and spat growth, settlement and metamorphosis were made and integrated during the summer of 1970 at Woods Hole.

Melampus bidentatus Say is a species placed in the family Ellobiidae of the subclass Basommatophora, which group is generally regarded as encompassing the most primitive living "lung-snails" of the class Pulmonata. As in all pulmonates, the molluscan mantle-cavity has been modified to a gill-less vascularized lung, and ellobiids are functionally "air-breathers." However, certain anatomical features, including many in the nervous, excretory and reproductive systems of ellobiids, remain considerably less specialized than those in more typical Pulmonata (Morton, 1955a, 1955b), and the Ellobiidae retain a number of features more diagnostic of archetypic marine gastropods. In the life-cycles of certain ellobiid species, including *Melampus*, a free-swimming veliger larva is retained, as in *no* other pulmonates. It should be remembered that molluscs are largely marine. Only certain gastropods (the class Pulmonata, and—particularly in the tropics—certain genera in four prosobranch superfamilies) are found on land, and they probably number less than twenty per cent of molluscan species (Thiele, 1931; Russell Hunter, 1964; Boss, 1971).

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[1] Supported by Grant GM11693 from the National Institutes of Health to W. D. Russell-Hunter, and Research Grant #1116 from the City University of New York to Martyn L. Apley.

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As in such forms as the "primitive" vertebrate lung-fishes, considerable significance attaches to any observations on the structural and functional adaptations shown by amphibious snails such as ellobiids. For *Melampus bidentatus*, this amphibious animal living in the highest tidal zone of salt marshes, we can now describe an elaborate succession of temporal adjustments of reproductive development and early growth which corresponds to the pattern of spring tide submergence. The difficulties of rapid larval and spat growth from a relatively small "primitive" egg (and therefore from a relatively small veliger at hatching) to a miniature version of the adult snail capable of surviving its first winter in the upper levels of the salt marsh have also been investigated. The rapidly changing growth patterns involve a major shift in shell growth (or a metamorphosis) occurring after settlement. In discussing these shell growth changes, we are able to present an unusually complete picture of development in a case of "heterostrophy" of larval and adult shell.

Such observations on amphibious forms like *Melampus*, and more particularly data on their physiological ecology, can have two kinds of significance. First, at the level of mechanistic physiology, they help establish our concepts of how such an "unlikely" animal machine as one built on the basic molluscan plan—involving elaborate ciliary, mucous, and hydraulic mechanisms of great efficiency in an aquatic environment—can maintain itself on land. Secondly, it is possible that hypotheses on the evolution of the major land stocks can be modified as a result of physiological and ecological investigations of amphibious stocks. No one would claim that contemporary ellobiids were descended unchanged from the stocks ancestral to the present 8000 species of efficiently terrestrial Pulmonata, but they undoubtedly face the same physiological problems and live in the same variable habitats as did the actual Jurassic ancestors of these most successful of land snails.

Observations and Results

I. Eggs and egg-laying

The egg-masses are gelatinous and have no capsules or protective coatings such as are found in the majority of pulmonate snails. On laying, they are usually irregular hemispheres of 1–2 mm diameter, approximately 0.5 mm thick in the center. Each egg-mass is made up of from 539 to 1240 eggs (mean = 850 for 42 egg-masses counted) which are deposited in a single continuous strand consisting of evenly-spaced eggs (each 170 μ long, see Fig. 1A) enclosed in two tubular gelatinous layers.

The inner thicker material, concentrated primarily around the eggs but continuous within the egg-strand, can be referred to as the spherical envelope and is surrounded by the outer more tubular layer termed the string membrane (Fig. 1A). There is no direct evidence of the specific origins of these elements in different parts of the female genital tract, but it is tempting to suggest that the spherical envelope, which must be laid down first, is secreted by the albumen gland along with the egg-shell itself, and that the string membrane has its origin in the mucous gland. As described by Apley (1970), the ova in *Melampus* almost certainly traverse a channel running through the mucous gland prior to egg-laying. It would be misleading to attempt to homologize these two layers in

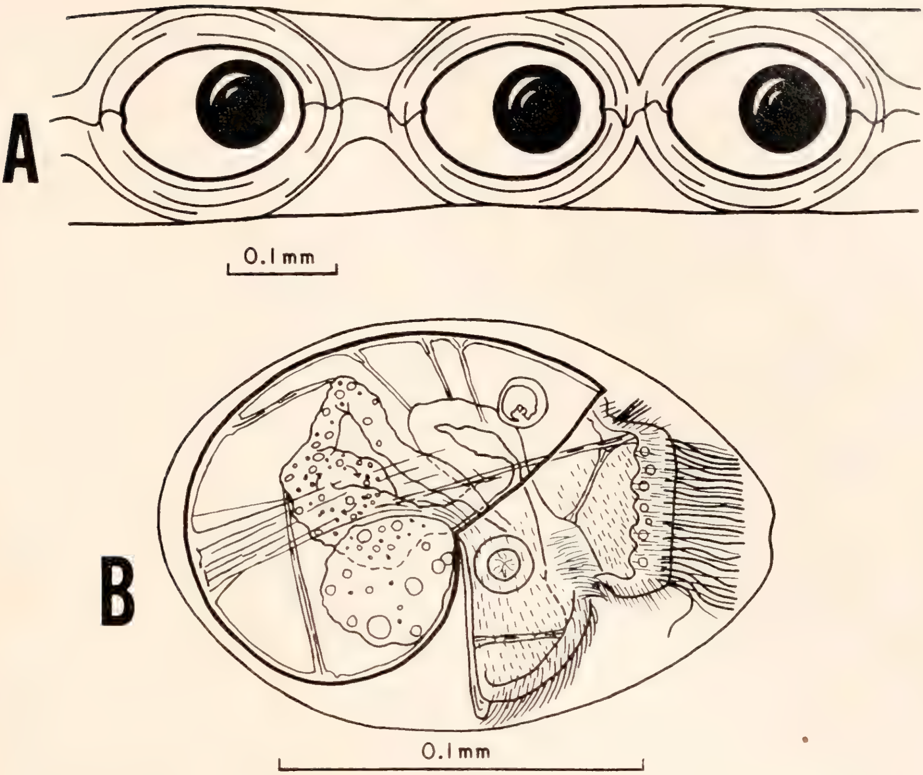

FIGURE 1. Eggs of *Melampus bidentatus:* (A) part of an egg-strand from a newly laid egg-mass, showing the two tubular gelatinous layers surrounding the eggs; and (B) an egg after 11–12 days of development containing a well-differentiated and active veliger larva.

Melampus with the detailed nomenclature set up for the higher limnic pulmonates by Bondesen (1950), but it is worth noting that no pedal glands are involved in this egg-mass secretion. Among the higher limnic Basommatophora, "external" secretions from the foot are important as the origin of the tough outermost coatings of the egg-capsules in such forms as ancylid limpets (Russell-Hunter, unpublished). The tubular string membrane in *Melampus* appears to form the general jelly matrix of the egg-masses as they "age" during the first days after laying, and is usually indistinguishable by the time of hatching. The spherical envelope, although continuous in the egg-strand, is concentrated primarily around the egg-shells themselves, appearing as optically distinct fine concentric bands in life, with lesser amounts in each "neck" between eggs. Within this, and connecting each egg with the preceding egg is a fine string-like structure, termed the allochalazal strand, which is probably continuous with the outer portion of the egg-shell. Attached at the points of indentation in the ends of the egg-shell (Figs. 1A and B), the allochalazal strand has been found to be mechanically important in the process of hatching which is described in the following section.

The organic content of the egg-mass layers (excluding the eggs themselves) is relatively low and it is obvious that free water is taken up at oviposition. Egg-masses are never laid in totally dry conditions but, in contrast, adult snails are never submerged when egg-laying. The ecological and behavioral implications of this have been outlined before (Apley, 1970; Russell-Hunter *et al.*, 1970) and will be discussed in more detail below. Some biomass values can be given for the individual eggs. Mean dry weight is 354 ng (mean for 18 batches) and an average wet weight would be nearly 13 times greater at 4.72 μg (mean of 10 batches totalling 8,909 eggs). Wet weights of eggs are much influenced by any adherent envelope and membrane material from the mass, and attempts to standardize wet weights of entire egg-masses failed. In fact, a series of experiments with closed dishes at different relative humidities, showed that entire early egg-masses behaved like hygrometers in their relatively rapid weight responses. Similarly, submerging early egg-masses in waters of salinity range 25%–100% SW produced weight changes indicating that the masses were behaving like near-perfect osmometers. Both in the field, and in laboratory cultures, the egg-masses remain stiff turgid hemispheres for only about 3–4 days out of the 12–13 of development, and become more flaccid and irregular for the later two-thirds of the time until hatching. Total organic carbon content (determined by a wet oxidation technique: Russell-Hunter, Meadows, Apley and Burky, 1968) gave mean values of 109 ng carbon per egg (mean of 13 determinations totalling approximately 171 mg wet weight of eggs). Total nitrogen content (determined using a modified micro-Dumas technique on a Coleman semi-automatic nitrogen analyzer) was equivalent to 23.9 ng nitrogen per egg (mean of 9 determinations totalling approximately 14,000 eggs). In bioenergetic terms, these are remarkably small eggs for pulmonate snails. Comparable mean values for individual eggs in the freshwater limpet, *Laevapex fuscus*, are 4.31 μg C and 1.35 μg N (McMahon, 1972) and for *Lymnaea palustris* 25 μg C and 3.61 μg N (Hunter, 1972). Actual egg production by *Melampus* was most carefully assessed in 1966, when the annual reproductive period encompassed three semilunar cycles of egg-laying (Apley *et al.*, 1967; Apley, 1970). Before reproduction began, groups of 10 to 58 snails were isolated in the laboratory in eight mass culture dishes and maintained in appropriate conditions of light and temperature on damp filterpaper. No attempt was made (or was needed, see below) to simulate tidal conditions. The observed groups totalling 244 mature snails laid 219×10^4 eggs, then 345×10^4 eggs, and finally 254×10^4 eggs. Therefore, the overall fecundity totalled 818×10^4 eggs, corresponding to an average of 33,150 eggs per snail per year, laid in an average of 39 egg-masses. Thus, despite the comparative minuteness of the individual eggs, the reproductive period represents a period of exceedingly high bioenergetic output for mature specimens of *Melampus*. Elsewhere (Apley *et al.*, 1967) we have computed that 87% of the non-respired assimilation (N-RA) is directed to egg-output during the reproductive period, corresponding to 46% of the total annual N-RA, or to 32% of the N-RA if spring pre-breeding growth rates were sustained throughout the year. This output per individual standard snail corresponds to 7.3 mg dry organic material annually. As is the case in the few invertebrate species yet studied bioenergetically, much of the reproductive output must be sustained by the concurrent food input being at a relatively high level for the species. However, in this work we noted a gonadal depletion during the three cycles of the reproductive period amounting to 78% of the initial organic carbon content and to over 91% of the total nitrogen corresponding to a change of the C:N ratio of the gonad tissues from 6.3:1 to 15:1 (figures recalculated from data summarized in Apley *et al.,* 1967). This led to the hypothesis (Russell-Hunter, 1970) that egg-production was limited by the rate at which suitable organic nitrogenous materials could be made available to the gonad, in a fashion analogous to inorganic nitrogen as a limiting factor for the primary productivity of certain ecosystems.

As will be discussed, several aspects of survivorship during the early life-history of *Melampus* are related to this high numerical fecundity and the considerable bioenergetic output which it demands.

For obvious adaptive reasons, egg-laying by natural populations of *Melampus* takes place within about four days in each two-week cycle when their habitat in the upper salt-marsh is flooded by spring high tides (see Figs. 8 and 10). During the reproductive period of summer 1970, for example, there were four cycles of egg-laying at semilunar intervals in late May, June and early July. As we have confirmed in several summers (1965, 1966, 1967, 1968, 1970), stocks of *Melampus* brought into the laboratory maintain through the summer reproductive period the same pattern of semilunar egg-laying in the total absence of all tidal stimuli. There are other data (see Apley, 1970; Russell-Hunter *et al.*, 1970) which confirm that laying of egg-masses in phase with spring high tides is an obligate process. Physiological and ecological implications of this will be discussed in Section IV.

Under field conditions, this periodicity of egg-laying ensures that immediate desiccation of freshly laid egg-masses cannot occur. As first noted by Apley (1970), an even more important result of concurrence with the high spring tides is that the tidal flow through the "meadows" of *Spartina patens* and *Distichlis spicata* in the high salt-marsh (see Section IV, below) causes a redistribution of fine detritus and organic debris among the stems and bases of the plants. This detritus sifts over and covers the egg-masses with a layer which collects and maintains moisture around the eggs through the ensuing cycle of neap tides. In the field, undoubtedly it is the conditions ensured by this detritus layer which permit the survival and development of the eggs through the 11 or so days of nonsubmersion until the next spring high tides occur.

In the course of experimental work on hatching conditions (Section II, below), egg-masses were maintained on moist filterpaper in petri dishes stored at constant temperatures of either 18° C or 25° C. Development times did not vary greatly over large numbers of culture dishes. At 18° C, first cleavage takes place 5–7 hours after the egg-mass is laid, there is an early blastula by 12 hours, a true trochophore by the fourth day, and a well-differentiated and active veliger (Fig. 1B) by the eleventh day. Corresponding times at 25° C are trochophores by the third day, and active prehatching veligers by the ninth day.

II. Hatching, natural and experimental

In field populations of *Melampus*, the great bulk of egg-masses hatch about 13 days after laying when, in the natural course of the lunar cycle, they are once again subjected to several successive submergences. At these spring high tides, enormous numbers of newly hatched veligers (Fig. 2C) can be collected in the plankton of the seawater flooding the marsh. This massive and synchronous hatching of free-swimming veligers from the egg-masses is the second event in the early life-history of *Melampus* to be confined (again for obvious adaptive reasons) to about four days in phase with spring tides. The rigid pattern found in synchronous egg-laying raises the question of whether the similarly synchronized hatching is obligate (dependent on an innate and rigorous developmental timetable) or facultative (dependent on the incidence of tidal submergence).

This question was the basis for an extensive series of laboratory experiments in summer 1970, and these demonstrated that there is greater potential flexibility in the time of hatching than could be deduced from the field data alone. Egg-masses laid in the laboratory were set out on moist filterpaper in petri dishes usually within six hours after laying. The filterpaper was moistened with a 50:50 mixture of distilled water and filtered seawater (corresponding roughly to the tonicity of the soil water in the high salt marsh during the days between spring tidal flooding). Care was taken to avoid having any excess water in the dishes (by daily checking) during their storage at constant temperatures of either 18° C or 25° C. Hatching of free-swimming veligers can normally take place only when masses containing appropriate developmental stages are flooded with seawater. The matrix jelly of egg-masses with eggs in *earlier* developmental stages has been found to behave almost like a perfect hygrometer and osmometer, swelling by uptake of free water especially if it is of reduced salinity, and shrinking proportionately upon desiccation. At later developmental stages (around 7.5–10 days at 18° C) flooding will cause premature eclosion (abortion) of larvae from a varying proportion of eggs in the mass. These larvae are not fully differentiated free-swimming veligers and their survival is doubtful. For still older masses, where complete flooding would produce successful veliger hatching, smaller amounts of ambient water in contact with the egg-masses (for example, a thin film of free

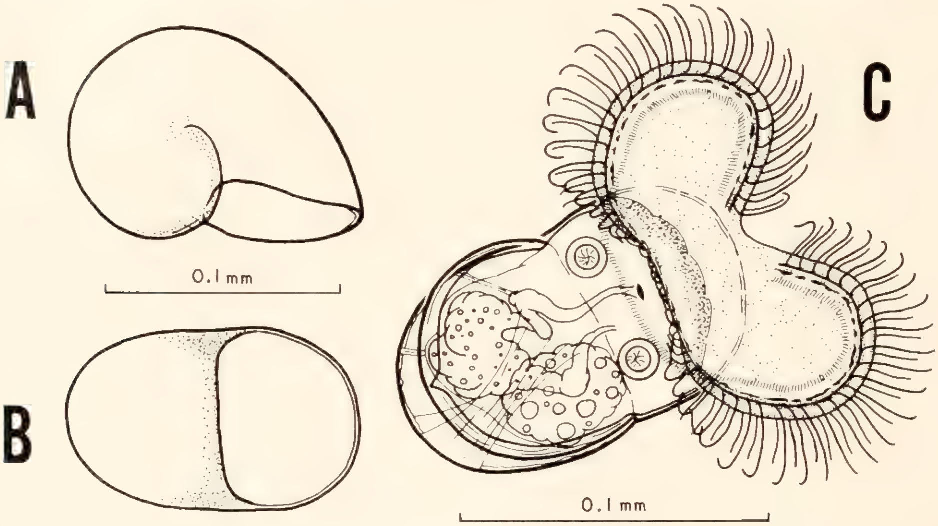

Figure 2. The veliger larva of *Melampus* at hatching: (A) and (B) two views of the simple planospiral veliger shell; and (C) a ventral view of a living veliger. For further description, see text.

water over the filter paper in our petri dishes) will produce eclosion of veligers which then lie inactive on the jelly surface but can survive for a number of days (probably at least four) to become active and free-swimming upon appropriate flooding.

Apart from these preliminary timings and observations, our more quantitative experiments fall into three groups (Tables I–III). In all three series, numbered egg-masses were set up in petri dishes shortly after laying. Earlier laboratory observations had established that, although egg-masses can be deposited at all times of the day and night, about 90% are laid between midnight and 8 AM. Examination by day of egg-masses laid in the field on the previous night reveals a majority of early cleavage stages with some blastulas, which suggests that the diurnal pattern of oviposition observed in the laboratory also occurs in natural field populations. The majority of the hatching experiments were set up around 10 AM when egg-masses laid since the previous midnight were screened and all showing later cleavage stages (or blastulas) discarded, along with any containing large percentages of abnormal, infertile or uncleaved eggs. Thus we began with each experimental group consisting of viable eggs at approximately the six-hour stage of development (or with a modal laying time of 4 AM). Normally 10, 20 or 30 egg-masses were set out for each time-temperature category in each experiment, and it should be noted that the percentages of hatching recorded in the tables were derived from a series of observations on *each* egg-mass which consisted of some 600 to 1100 eggs. Each record of percentage hatching for an egg-mass was based on about four detailed assessments of areas using a medium-power (× 45) dissecting microscope along with an inspection of the entire egg-mass. At times eclosion was seen to begin in one distinct zone of the egg-mass.

TABLE I

Experimental hatching of Melampus *eggs: age at hatching of 18° C and 25° C stocks with single flooding*

Series	Date laid	No. of egg masses	Temp. (°C)	Date of flooding	Age at time of flooding (days)	Mean percentage hatching					Age at 90% hatching (days)
						2 hrs	8 hrs	24 hrs	28 hrs	48 hrs	
18/10M/A,F	7/10	20	18	7/17	7.3	0	0	0	20	55	9.3
25/10M/C,J,P	7/10	30	25	7/17	7.3	1	1	5	—	98	9.3
18/09M/A,F,M	7/09	30	18	7/17	8.3	0	0	0	10	73	10.3
25/09M/C,J,P	7/09	30	25	7/17	8.3	0	0	0	10	98	10.3
18/08M/A,F,M	7/08	30	18	7/17	9.3	0	0	0	10	98	11.3
25/08M/C,J,P	7/08	30	25	7/17	9.3	0	0	1	–	98	11.3
18/10M/B,G	7/10	20	18	7/20	10.3	0	0	0	94	–	11.5
25/10M/D,K,Q	7/10	30	25	7/20	10.3	4	13	30	93	—	11.5
18/09M/B,G,N	7/09	30	18	7/20	11.3	0	0	0	95	—	12.5
25/09M/D,K	7/09	20	25	7/20	11.3	1	5	6	94	—	12.5
18/08M/B,G,N	7/08	30	18	7/20	12.3	0	0	0	98	—	13.5
25/08M/D,K,Q	7/08	30	25	7/20	12.3	3	7	33	98	—	13.5

The first series of experiments, using egg-masses cultured both at 18° C and at 25° C establish the minimal ages at which viable veliger eclosion can occur as a response to sustained flooding with seawater. The earliest successful hatchings in our material (see Table I) were from masses kept at 25° C where 98% hatching was achieved after 48 hours of flooding. These egg-masses were then at about 9.3 days development. At the more "normal" temperature of 18° C, > 90% hatching occurred after 48 hours of flooding in a few cases at developmental "age" of 10.3 days and more generally in cultures which reached 11.3 days at the end of the 48 hour period of flooding. Universally in cultures from both 18° C and 25° C > 90% hatching was achieved where the terminal "age" lay between 11.5 and 15 days.

Of course, such laboratory experiments where hatching resulted after a period of *continuous* flooding with seawater are somewhat unnatural. Two series of experiments (Tables II and III), more closely reflecting field conditions, involved "tidal bathing," in which the egg-masses were submerged for successive periods of one hour at intervals of approximately 12.5 hours (corresponding to successive spring high tides). The second series of experiments utilized two short (1 hour) floodings at tidal intervals (12.5 hours) followed by a third flooding continued to > 90% hatching (Table II). In the majority of cases (at a variety of ages from 13 to 16 days, and from development at 18° C and 25° C), > 90% hatching of veligers had occurred by 28 hours submergence in the "third flooding."

TABLE II

Experimental hatching of Melampus *eggs: effects of two advance "tidal" floodings at intervals of 12.5 hours* (2AF) *compared with control groups of the same ages*

Series	Date laid	No. of egg masses	Temp. (°C)	Treatment	Age at time of first flooding (days)	Mean per cent hatching after first flood	Mean per cent hatching after second flood	Mean per cent hatching following third and continuous flooding				Age at >90% hatching (days)
								2 hrs	8 hrs	24 hrs	28 hrs	
AF18/10M/HO	7/10	20	18	2AF	12.3	0	0	20	30	68	93	14.5
18/10M/E	7/10	10	18	control	13.3	0	0	0	0	0	90	14.5
AF25/10M/LR	7/10	20	25	2AF	12.3	0	2	98	—	—	—	13.4
25/10M/I	7/10	10	25	control	13.3	0	0	5	30	50	90	14.5
AF18/09M/H	7/09	10	18	2AF	13.3	0	0	20	20	80	98	15.5
18/09M/EO	7/09	20	18	control	14.3	0	0	0	0	1	90	15.5
AF25/09M/L	7/09	10	25	2AF	13.3	0	25	98	—	—	—	14.4
25/09M/IR	7/09	20	25	control	14.3	0	0	5	35	65	90	15.5
AF18/08M/HO	7/08	20	18	2AF	14.3	0	0	35	45	98	—	16.3
18/08M/E	7/08	10	18	control	15.3	0	0	0	0	1	90	16.5
AF25/08M/LR	7/08	20	25	2AF	14.3	1	50	98	—	—	—	15.4
25/08M/I	7/08	10	25	control	15.3	0	0	30	30	85	90	16.5

The third series of experiments was closest to field conditions, and in it the egg-masses were again submerged for four successive periods of one hour at intervals of 12.5 hours. Timings were arranged so that the fourth floodings corresponded to the twelfth to fourteenth days of development, and only healthy egg-masses kept at 18° C were used (Table III). Under these conditions, the first and second floodings had no overt effect and the third flooding produced

< 5% or *no* veliger hatching regardless of "age." In cases where an age of 14 days had been reached, the fourth flooding was usually completely effective and always produced appreciable hatching in 30 minutes and > 98% hatching if allowed to continue for 1.5 hours. There was a tendency where ages of 12 and 13 days had been reached for hatching to be less complete (< 50%–85%) and to take somewhat longer (> 2 hours) in the fourth flooding. It should be noted that the total time immersed in water in these experiments (for nearly complete hatching at age of 14 days) is only 4.5 hours. This is less than one-tenth of the required time submerged (48 hours) in the experiments involving sustained flooding, and much more closely comparable to natural tidal conditions in the field. However it should also be noted that the elapsed time in *all* the successful experiments was about 48 hours.

TABLE III

Experimental hatching of Melampus *eggs: effects of four successive "tidal" floodings each of one hour's duration*

Series	Date laid	No. of egg masses	Temp. (°C)	Age at first flooding (days)	Mean percentage hatching			Mean percentage hatching, fourth and continuous flooding, elapsed hours			Age at 90% hatching (days)
					first flooding	second flooding	third flooding	1 hr	2–3 hrs	9–12 hrs	
18/10M/S	7/10	10	18	10.5	0	0	1	17	90	90	12.0
18/10M/T	7/10	10	18	10.5	0	0	0	8	60	60	—
18/10M/U	7/10	10	18	10.5	0	0	0	18	75	75	—
18/10M/V	7/10	10	18	11.0	0	0	1	2	5	98	13.0
18/10M/W	7/10	10	18	11.0	0	0	1	2	5	98	13.0
18/10M/X	7/10	10	18	11.0	0	0	1	2	5	98	13.0
18/08M/S	7/08	10	18	12.5	0	0	4	20	98	—	14.0
18/08M/T	7/08	10	18	12.5	0	0	4	20	98	—	14.0
18/08M/U	7/08	10	18	12.5	0	0	4	35	98	—	14.0

The veligers in egg-masses raised in the laboratory proved to retain some viability over an extraordinarily long time span. Over-extension of a few experiments showed that viable veligers could still hatch from egg-masses maintained at 18° C 23 days after laying. Our most extreme record of egg-masses (maintained at 18° C but not carefully kept and probably somewhat dried out on occasion) yielded about 80% hatching of veligers after 44 hours of flooding and at 42 days after laying. This extraordinarily long viability could have some adaptational significance in natural populations. The highest level reached by successive sets of spring tides varies throughout the year and, in the habitat of *Melampus* (see Section IV), it would be possible for eggs to be laid after spring tide flooding at a level which would not be submerged in seawater again for 56 days (or four complete cycles of spring and neap tides). Obviously few adult snails are ever found at such levels of the salt marsh and fewer still will lay eggs there, but the flexibility of hatching age and the extreme viability of eggs after they have reached the veliger stage of development could accommodate such occurrences.

From all this, it is clear, that unlike the rigid semilunar pattern of egg-laying, the synchronous tidal occurrence of hatching in *Melampus* is not obligate. The developmental age at hatching is flexible—but eclosion will usually be brought about from egg-masses of 10 to 24 days by a *sequence of about four tidal floodings in under 50 hours.* Being facultative, the process allows better survival and overlap of cohorts but it also re-establishes the synchronization. As will be discussed later, this is highly significant for the future synchronous resettlement of veligers into the appropriate levels of the salt-marsh habitat at a later cycle of spring tides.

During the experimental work on hatching, details of the process of eclosion were followed on a number of occasions. Since the detailed papers of Davis (1964, 1967, 1968) on hatching processes in various invertebrate eggs do not cover any ellobiid snails, it is worth briefly reporting here on the mechanism of hatching in *Melampus.* The first visible sign of hatching is a wrinkling and loss of rigidity of the egg-shell where it overlies the active cilia of the velum of the contained larva. This wrinkling can begin at either the blunter or the more pointed end of the egg (see Fig. 1B) but is always initiated over the velum. The wrinkling seems to be caused by some process internal to the egg-shell, and could result from the detection by the contained veliger of some earlier stimulus to hatching. It is tempting to suppose that certain elapsed changes in the osmolarity of the jelly matrix of the egg-mass provide this stimulus. As the crumpling spreads and extends over the entire surface of the egg-shell, the velar end of the egg is ruptured by vigorous thrusts of the velum and head region. It is worth noting that, after the wrinkling has begun, all the subsequent stages of hatching can be explained as mechanical. The veliger is not able to move its cilia effectively until it has freed the velum of remnants of softened egg-shell fragments. Final separation of egg-shell and veliger is aided by the allochalazal strand, an anchoring device, which allows the now effectively swimming veliger to pull away from the clinging mass of egg-shell. Before hatching the allochalazal strand can be seen to run from one egg-shell to another between which it is embedded in the concentric membranes of the spherical envelope (Fig. 1A). The attached strand which survives the softening and crumpling process of early eclosion is, of course, at the opposite end of the egg-shell. Once free of the egg-shell, other envelopes and the jelly matrix offer little resistance to the veliger which, by vigorous movements of the operculum and beating of the velar cilia, can swim free of the egg-mass in less time than it took to escape the egg-shell. Hatched veligers then become relatively inactive unless there is a sufficient volume of seawater available for active swimming and feeding. Under natural circumstances, they swim actively and are swept out to sea from the salt marsh with the ebbing spring tide to become part of the inshore plankton.

III. Mantle-shell changes in early growth

On hatching, the relatively small "primitive" egg of *Melampus* yields a small veliger (Fig. 2C). Apart from the ecological difficulties and the synchronization of the shifts into and out of the salt-marsh habitat (Sections II and IV), there have to be major changes in the patterns of mantle-shell growth to accommodate the anatomical and behavioral transitions from ciliated planktonic larva to spat and young adult. The latter shift involves a true metamorphosis of mantle and shell.

This can be stated quite simply: the larval and postlarval shells in *Melampus* are sinistral in coiling and low-spired (nearly planospiral), while the spat and adult shells are dextral and truly turbinate. Snail shells showing evidence of such reversal of the spiral axis of growth have long been termed heterostrophic. There are a number of early records of heterostrophy in the Ellobiidae (Fischer and Crosse, 1900; Pelseneer, 1901, 1906; Harry, 1951; see also Morton, 1955a, b, and references therein), but only one illustrated description (Harry, 1951) of a single juvenile shell in a species of *Pythia* from the Philippines. We can now provide, for *Melampus*, the first detailed account of the stages of development of an ellobiid heterostrophic shell. The functional and ecological implications, both of the mantle-shell metamorphosis and of the other, more gradual, changes in growth gradients in early stages of *Melampus*, are not without evolutionary significance.

The newly hatched veliger of *Melampus* (Fig. 2C) has a shell of about 125 μ maximum diameter (Figs. 2A, B) which appears almost planospiral (that is, indistinguishable as regards sinistrality or dextrality because of the lack of an apex). As in the majority of snails which hatch as veligers, the mantle rudiment and the shell which it secretes are well-differentiated long before hatching. When eggs developing at 18° C are followed, we have a true trochophore by the fourth day and, within the next 24 hours, the first mantle rudiment becomes distinguishable on the visceral mass while the other half of the now "waisted" embryo differentiates into head-foot-velum rudiments. By the sixth day, when torsion has occurred, the mantle-shell rudiment is clearly defined and cup-shaped, and a tiny but obvious opercular rudiment appears on the posterior part of the foot. From the seventh to the tenth days, most active growth of the mantle occurs around that arc of its edge facing the velum, generating (by this simplest possible of pallial edge growth gradients) the planospiral shell (Figs. 1B and 2ABC) of the veliger at hatching. This veliger shell is almost entirely proteinaceous and little secretion of calcium carbonate has occurred. Veligers of mean tissue dry weight 129 ng had shell calcium carbonate values of less than 13 ng, which can be compared with early settled spat stages of *Melampus* where shell calcium makes up half of the dry weight (Russell Hunter and Apley, 1966).

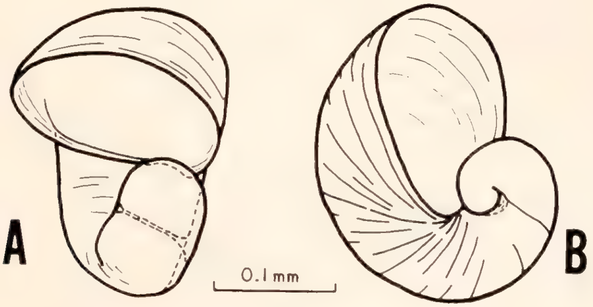

FIGURE 3. The shell of a veliger of *Melampus* after fourteen days of planktonic life. The shell coiling now appears moderately turbinate and sinistral, and the original prehatching shell can be distinguished in 3B. For further discussion, see text.

As the veliger grows in the plankton, the shell coiling becomes turbinate (that is, no longer has a spiral axis in one plane) and appears sinistral (Fig. 3AB). [Throughout this section, for convenience of description, the terms sinistral and dextral are used only in their long-established *descriptive* sense as applied to the external appearance of turbinate coiled shells. Thus sinistral refers to coiling which appears to grow anticlockwise when viewed from the point of origin, dextral to apparently clockwise growth. Their *morphological* sense, as regards the orientation of the heart and other asymmetric organs in gastropod anatomy, is not implied here. The metamorphosis which we are about to describe is one of shell-coiling reflecting a shift of the growth of the mantle from sinistrality to dextrality; it implies no changes in living structures other than the actively growing pallial edge; and it certainly does *not* involve shift to a condition of *situs inversus* for the heart, the kidney and all other unpaired structures. Our use of sinistral and dextral in these descriptions will be related to the morphologically more correct terms hyperstrophic and orthostrophic only in the final discussion.] The veliger shell illustrated in Figure 3AB is about 245 μ in maximum dimension, shows sinistral coiling, and has a rapidly enlarging aperture to accommodate the large lobes of the velum at this stage. Since the shell has "grown" by marginal increments laid down by the secretory cells of the mantle-edge, it is clear that we have had some *allometry* here in the growth gradient pattern of cell-divisions at the pallial edge. The shell of Figure 3AB is from a veliger of 14 ± 1 days after hatching, grown in a laboratory culture. Similar late veligers were found occasionally in the inshore plankton, but neither culture nor field material was available in sufficient quantity for any analyses of organic carbon or of shell calcium. However, manipulating these tiny shells suggested that some calcification had occurred since hatching. It is worth noting that veligers of this age retain the operculum which has become greatly enlarged since hatching. The majority of prosobranch snails have an operculum througout life, and even those with limpet-shaped shells and no operculum as adults, such as *Acmaea* and *Crepidula*, have a larval operculum as veligers. In the two subclasses of "higher gastropods," *all* adult pulmonate snails (and almost all opisthobranchs) lack the operculum. Only in a few ellobiid genera like *Melampus* is there a temporary operculum in the veliger stage. Although this may have some phyletic significance, it is worth putting forward a simple functional explanation for the retention of the operculum at this stage, again based on the need to accommodate the large velar lobes. For a non-operculate snail such as a pulmonate or shell-bearing opisthobranch to be fully protected by the shell, the head-foot must be withdrawn well beyond the edge of the shell at its aperture, and thus the internal volume or capacity of the shell must be considerably in excess of the total volume of the tissues. This need not be so in a snail with an operculum which can close to an accurate fit with the edge of the shell at its aperture. The fact that the adaptational significance of withdrawal to the veliger may involve the rapid "switching-off" of its locomotory cilia as well as "protection" (Garstang, 1928) merely emphasizes the advantages conferred by the operculate condition in terms of the economy of shell-growth. We have some records of veligers retained alive in culture (without settlement) for 20 days, by which time the shell had grown to a maximum of 276 μ though the latter part of the shell-growth involved the slower enlargement of the aperture typical of the postlarva and was unlike the earlier veliger "flare." We assume, however, that Figure 3AB is more typical of the form of a late veliger shell just before settlement.

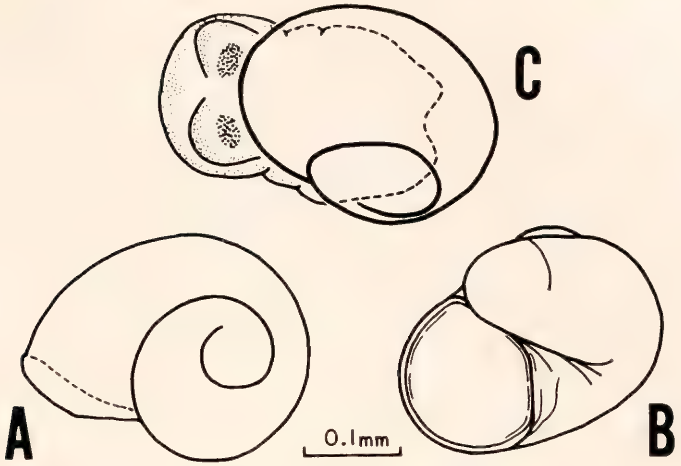

FIGURE 4. The "postlarva" of *Melampus* after settlement but before metamorphosis: (A) and (B) two views of the sinistral turbinate shell; and (C) a dorsal view of a living specimen collected in the salt marsh after settlement.

After this stage, settlement occurs (back into the salt marsh, see Section IV) and the postlarva quickly loses both velar lobes and operculum, as it takes up a crawling rather than a swimming mode. The maximum shell dimension at settlement lies between 270 μ and 290 μ. Shell metamorphosis does *not* occur at this time. The shell as it appears a few days after settlement is shown in Figures 4A and 4B. It is about 290 μ in maximum dimension, still shows sinistral coiling, but recent increments to the edge have involved *isometric* growth gradients in the mantle-margin (that is, during the last half-whorl of the enlargement of the shell and of the slower enlargement of its aperture, the *shape of the aperture has not changed*). The shell is now more obviously calcified, but again we have no quantitative analyses for this stage. A living specimen of this premetamorphic postlarval stage, collected in the field at Sippewisset, is illustrated in Figure 4C.

Soon after this, the metamorphosis in the growth of the mantle tissues occurs, the immediate result of which is the initial secretion of a spat shell which shows

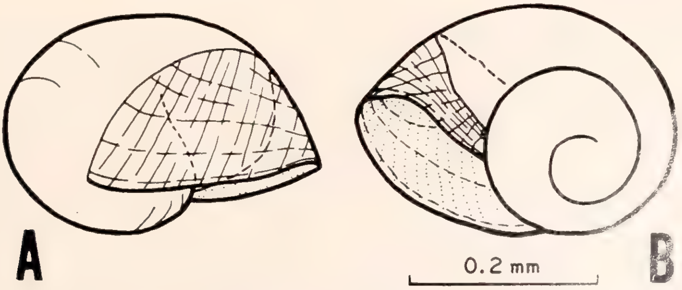

FIGURE 5. The first shell growth after metamorphosis of the mantle in *Melampus*. For further discussion, see text.

the new dextral coiling which will be retained throughout spat, and juvenile and adult growth (Figs. 5AB, 6ABC). The first shell secretion after metamorphosis is shown in Figures 5A and 5B. The reorientation of the spiral normal axis of growth can better be seen by comparing the slightly older postmetamorphic spat of Figures 6A and 6B, with the orientation of the earlier postlarval shell (or protoconch) at the apex of the later juvenile shell shown in Figure 6C. All these later illustrations (Figs. 4–6) are based on field specimens collected at appropriate times in Sippewisset marsh, and are previously undesscribed. Appropriate comparisons of these figures show the shift from a sinistral to a dextral shell-coiling. As has often been pointed out, the growth and subsequent configuration of the gastropod shell can be conceptualized as consisting of two somewhat distinct growth processes (Huxley, 1932; Russell-Hunter, 1953a, 1968; Fretter and Graham, 1962; Wilbur and Owen, 1964). One is growth along a spiral normal axis which can be visualized as running from the origin of the spiral (the apex of the shell in most cases) to the centroid of the plane of the aperture of the shell (sometimes more loosely defined as the midpoint of the foot). The other is the continued expansion of the mantle (and therefore of the shell which it secretes) by marginal increments to accommodate growth of the body. As a result of this accretionary method of growth, the form of the shell may be described in terms of the growth gradients of the mantle edge which usually are consistently maintained through each period of growth. Biologically, of course, the twofold conceptualization is somewhat false: the properties of the

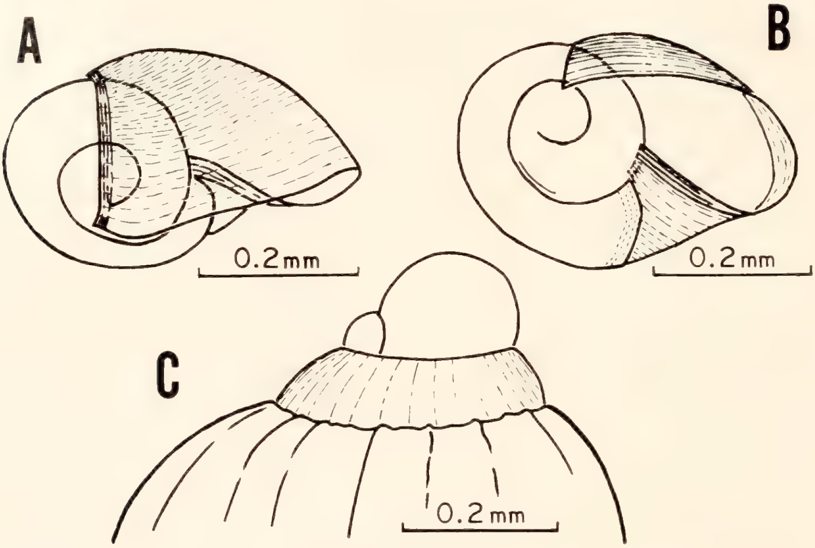

FIGURE 6. Metamorphosis of mantle-shell in *Melampus:* (A) and (B) two views of the shell of a postmetamorphic spat (slightly older than that illustrated in Fig. 5); and (C) the postlarval shell or protoconch at the apex of a later juvenile shell. The sinistral growth of the postlarval shell is replaced by a dextral shell coiling which persists in juvenile and adult. For further discussion, see text.

spiral normal axis (including the apical angle of its visualized cone) are not entirely independent of the growth gradients of the pallial aperture. Both are generated by the differential rates of cell division in the cells of the mantle edge.

Metamorphosis in *Melampus* involves an abrupt change by about 90° in the *direction* of the spiral axis and the initiation of an *entirely new pattern* of growth gradients for the mantle-edge (the new pattern being a slightly distorted mirror-image of the older one). All the other changes of shell shape in *Melampus* reported and illustrated here can be described in terms of sustained gradients between the rates of growth at points around the mantle-edge (allowing the aperture shape and the proportions between major dimensions to remain unchanged with growth, or isometry) or in terms of proportionally changing gradients between these rates (or allometry, as in the shell growth of the veliger after hatching). Such description of the mantle-shell metamorphosis in *Melampus* is not possible, and the

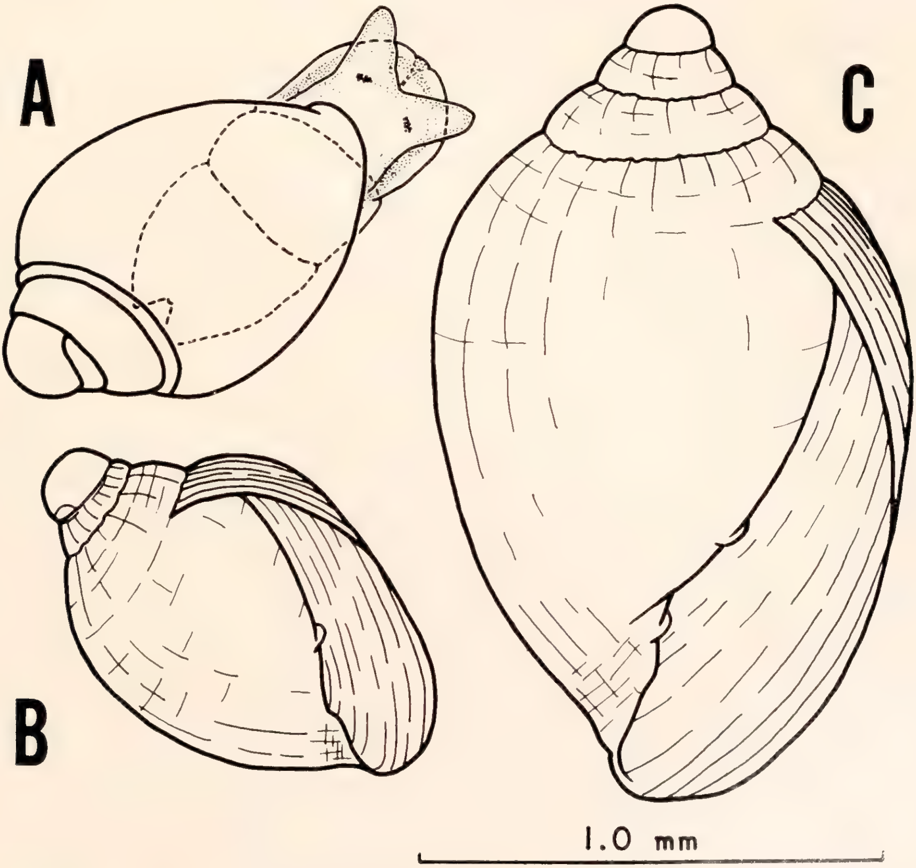

FIGURE 7. Later spat stages of *Melampus:* (A) dorsal view of a living specimen of a young spat about 4 to 5 weeks after settlement; (B) the dextral turbinate shell of a similar spat; and (C) shell of a spat about 8 weeks after settlement, in which the adult aperture shape (reflecting the growth gradients of the mantle-edge) is already established. Later shell growth is isometric, see text.

metamorphic process involves an abrupt change in the form of the annulus of dividing pallial cells which generates both differential shell increments and the growth spiral.

Soon after metamorphosis, a near-adult pattern of growth gradients is established in the mantle-edge. This can be seen by comparing the shell aperture in Figure 7C, of a 1.6 mm spat where the adult shell shape is already established, with the 400 μ postmetamorphic young spat of Figure 6B. Despite the fourfold increase in linear dimensions the shape generated by the pallial edge does not change: later shell growth is isometric.

A living young spat of about 1 mm shell-length collected in the field about four to five weeks after settlement is shown in Figure 7A. Batches of closely comparable spat, though somewhat smaller at a mean shell-length of 675 μ, provided some biomass data. Mean tissue dry weight is about 11.3 μg (mean for 8 batches) and mean organic carbon 5.03 μg (mean of 6 determinations), values about a hundred times those already reported for newly hatched veligers. As pointed out in an earlier note (Russell Hunter and Apley, 1966), in *Melampus*, growth (in any real biomass terms) extends through three orders of magnitude in the first three months of life and through nearly six in the entire 3–4 year lifespan. In the spat of mean length 675 μ the shell is now well-calcified with mean calcium carbonate at 11.9 μg (mean of 8 determinations) or over 50% of the dry weight (shell included). Similar cases of a shift from a non-calcareous and largely proteinaceous shell in the veliger to a largely calcareous shell in settled spat for marine prosobranch snails are discussed by Fretter and Graham (1962). Further aspects of the growth of spat and of their population dynamics will be set out in Section IV.

IV. Ecology of early life-history

Zonal distribution. Natural populations of *Melampus* are found in the higher levels of salt marshes. The zone, within which living animals are numerous and where all reproduction occurs, can readily be defined in terms of vegetation and soil, or in terms of the vertical excursion of the tides.

The natural vegetation zones in the upper levels of the salt marsh at Little Sippewisset are characteristic of those found throughout New England (New Jersey to Maine). Some marshes, where populations of *Melampus* have been studied by us, are much more extensive (for example at Lawrence, Long Island and at West Barnstable, north Cape Cod) but, although the zones of plants are of much greater horizontal extent in such marshes, the vertical distribution of plant species conforms to much the same pattern (see Chapman, 1940; Blum, 1968). At the landward edge of the marsh are stands of *Phragmites communis* and *Typha angustifolia* (cat-tails) with variable amounts of woody xerophytic bushes, such as *Iva frutescens* var. *oraria* (marsh-elder), *Myrica pensylvanica* (bayberry) and *Prunus maritima* (beach-plum). *Melampus* rarely moves into, and must never linger in, this zone. Woody glasswort (*Salicornia virginica*) occurs just below this level, fiddler-crabs (*Uca pugnax* and *U. pugilator*) are often abundant, and this is the uppermost level for populations of *Melampus*. There is next a patchy intermediate zone of the high-water species of "soft" marsh-grass, *Spartina patens*, of the rush *Juncus gerardi*, and of the spike-grass *Distichlis* *spicata,* the former two species covering the better drained patches. Then there is a slightly lower, slightly wetter, zone where *Distichlis* is dominant, with some *S. patens* and with occasional clumps of the rush, *Juncus gerardi,* and of another glasswort, *Salicornia europaea.* In this zone, the soil may still dry out sufficiently for *Uca* spp. to burrow. In this *Distichlis* zone, as in the *S. patens-Juncus-Distichlis* zone above it, *Melampus* is abundant, and it is largely in these zones that copulation and egg-laying occurs at appropriate spring high tides. As discussed in detail by Blum (1968), it is in these zones that the algal community of the soil surface is characterized by the dominance of a single species of *Calothrix.* Below these zones, and in clearly wetter conditions where the soil water is near the surface even at low tides and there are often superficial puddles, is a zone of the "dwarf" growth form of the major marsh-grass species, *Spartina alterniflora. Melampus* occurs in this zone, but does not normally lay eggs in the wetter, lower parts of it. As noted above, it is in this zone that spat of *Melampus* may seem to be most numerous during the first four to six weeks after settlement. Lower still are the taller stands of *Spartina alterniflora,* mostly three to four feet in height, principally along the edges of the small drainage channels and larger tidal creeks which dissect the march. *Melampus* does not occur in the drainage channels (or at any *lower* tidal level), and is only rarely found among the roots of the tall form of *S. alterniflora.* As this present manuscript was being revised, Redfield (1972)

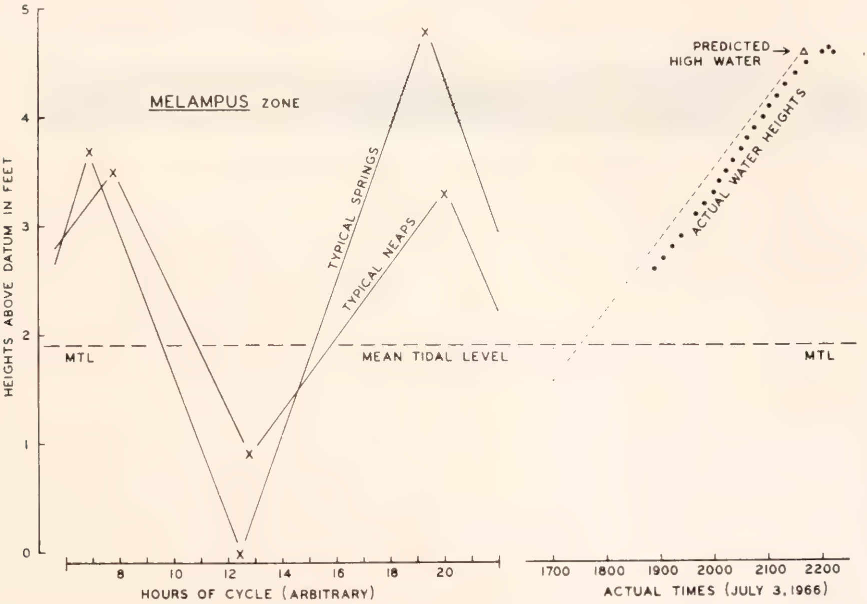

FIGURE 8. The relation of the vertical zone (3.9 to 4.4 feet above datum) occupied by natural populations of *Melampus* at Little Sippewisset, typical ranges of spring (−0.02 to 4.77 feet) and neap (0.9 to 3.28 feet) tides, and the observed time course of actual tidal heights during one high water of springs. Note that the mean level of all tides (MTL) does not necessarily correspond to mean sea level (MSL).

provided a detailed account of the ontogeny and plant ecology of the salt marsh at West Barnstable.

In summary, in terms of plant zonation, the populations of *Melampus* are found principally in the *Spartina patens-Juncus-Distichlis* zone, in the *Distichlis* zone, and in the upper levels of the zone dominated by the "dwarf" growth form of *S. alterniflora.* Under slight differences of drainage, the populations of *Melampus* can extend a few centimeters (actually about 0.25 feet) vertically above and below these zones. (In many marshes, of course, a few centimeters vertical extension could imply the horizontal colonization of hundreds of meters of marsh.)

Figure 8 shows the relationship of the zone occupied by *Melampus* at Little Sippewisset to the time course of the upper part of a spring tide cycle. In general terms, the range of normal spring tides will encompass all but a small number of extreme tides (perhaps all but six cycles in any year). If that range of springs is arbitrarily divided into eight zones of equal vertical extent—with four of them lying above the mean level of all tides (which does not necessarily correspond to mean sea level), then extensive populations of *Melampus* are almost entirely limited to the uppermost of these vertical zones (that is to the upper quarter of the shore lying above mean tide level). *Melampus* may extend downward through the next highest level, and upward to the extreme upper level bathed by *any* tides, but it is only abundant in, and only reproduces in, that uppermost eighth of the normal spring tidal range (or, at Little Sippewisset, 3.9 to 4.4 feet above datum). The figure shows the predicted curves for typical high tides of neaps and of springs at Sippewisset, and the observed water levels corresponding to the latter. On the evening of the observations, offshore winds caused a delay of about twenty minutes in the rise, but no apparent distortion.

Another way of putting it is that the populations of *Melampus* are largely found in the upper two thirds of the zone lying above the mean high water of neap tides (MHWN) and below the mean high water of spring tides (MHWS), but are also found in the zone lying between MHWS and the extreme upper limit washed by any tides. Thus they live in the upper half of what has been termed for rocky shores the *supralittoral fringe*, and defined as "the region within the littoral zone not wetted by all tides" (Stephenson and Stephenson, 1948; Southward, 1958, Table I, page 141).

Egg, larval and postlarval ecology. Our populations of *Melampus* live in the upper 12% of the intertidal zone, and some may be bathed by seawater for only 8 hours out of the 354.4 hours (or 2.3%) of each semilunar tidal cycle. Adults of *Melampus* do *not* migrate down to lower zones of the littoral for reproduction, as do certain arthropods of the supralittoral fringe. *Melampus* fits its peculiar environment by achieving strict synchronization with spring high tides for its processes of (a) copulation and egg-laying, (b) hatching, and (c) veliger settlement, and possibly some looser synchronization of intermediate stages of the life-history such as postlarval metamorphosis and early spat growth. These adaptational achievements of temporal "fitting" are the dominant aspect of behavior and ecology in *Melampus.*

As first elucidated by Apley (1967, and unpublished), the onset of the overall period of reproduction for these populations of *Melampus* in late-spring–early-summer is determined by changing day-length as one *essential* signal in a complex environmental input. This input includes conditioning temperatures and individual snail biomass with nutritional state, as well as day-length. In the laboratory, Apley (1967) was able to induce copulation and egg-laying in cultures during the month of January, by holding them at 22° C and applying day-length conditions of L16D8 and L14D10. Application of L12D12 under otherwise identical conditions did not induce reproduction. In these experiments there was a lag, or latent period, of about 20 days.

For the latitude of our populations at Sippewisset, the day-lengths of 12, 13 and 14 hours (L12D12, L13D11, and L14D10) are reached in spring on March 17, April 7 and April 30, respectively. Were it not for the importance of field temperatures, we might postulate that the field onset of reproduction might be set by the date of April 7 with a latent period of about 27 days (the extra 7 days to approximate the fit to the semilunar cycle). Thus theoretically, if field temperatures were around 18–20° C, we might expect the earliest reproduction around May 4. In fact, the field temperatures for the months of April and May at Sippewisset range from 3 to 13° C and from 6.5 to 19° C, respectively, and our earliest field observations of the aggregations, which precede copulation and egg-laying, have been on May 26 (1968) and on May 24 (1970).

In different years, the field onset of reproduction at Sippewisset has come at an appropriate day of spring tides in late May or early June. There are normally three cycles of egg-laying (occasionally four, see Table IV) in each annual reproductive period (Apley, 1967, 1970; Apley *et al.*, 1967). Thus this annual period always extends from late May or early June through early July.

Each breeding cycle within the reproductive period shows a definite semilunar periodicity with egg-laying confined to four days in phase with the spring tides. Taking full or new moon as day 0, the patterned behavioral sequence involves aggregation (day −1), copulation (day +1), egg-laying (days +2 through +6), and dispersion (days +6 through +8). This is shown as part of Figure 10. As already noted, stocks of *Melampus* brought into the laboratory from April onwards will maintain the same semilunar pattern of reproductive behaviour as the undisturbed field populations at Sippewisset.

In the reproductive period of 1970 in the salt marsh at Little Sippewisset, there were four cycles of egg-laying around the modal dates of May 24, June 7, June 22, and July 6. Each of these dates is about three days after a new or a full moon (that is, day +3 in the behavioral schedule set out above). The third cycle of egg-laying (around June 22) was considerably less productive than the massive egg-layings of June 7 and July 6.

Peak natural hatching of veligers occurred in the field with the spring tides of about 13 days later, actually on June 6, June 20, July 5, and July 19. Our regular townet collections were taken in the creek which drains the salt marsh during the two hours immediately after high tide, and thus they sampled the veligers being swept out to sea from the marsh. Such veligers were invariably newly hatched; older veligers were never collected at such times. The lunar phases in 1970 corresponding to these hatchings were new moon on June 4, full, June 19; new, July 3; and full, July 18. Some egg-masses must have remained unhatched in our field areas, because later plankton collections allowed us to deduce that some hatching occurred around August 2 (new moon), and we collected some apparently newly hatched veligers in the field on August 16 (full moon, August 17). This shows that the extraordinary viability of egg-masses in the laboratory hatching experiments (Section II), when kept out of free water for 23 and 42 days, is not artificial but parallels field conditions. It is unfortunate for our hypothesis that such egg-masses showing delayed hatching must have been laid in the highest levels of the marsh. While some of the highest spring tides of the entire summer came with the full moon at August 17, the spring tides of the new moon at August 2 were less extensive than any from May 1 through September 1, 1970.

Since the hatched veligers drift out into the general inshore plankton, we have been unable to establish the earliest larval growth rates from field samples. In laboratory cultures, the veliger of *Melampus* first feeds within one hour of hatching. As in the better known prosobranch veligers (Fretter and Graham, 1962; Fretter, 1967; Mapstone, 1970; Pilkington and Fretter, 1970) the same action of the preoral velar cilia propels the larva forwards, collects particulate food, and concentrates the particles in the food-groove. Both the preoral band of longer cilia (derived from the prototroch of the earlier trochophore larva) and the postoral band of shorter cilia (metatroch of the trochophore), which lie on either side of the food-groove, are essential to the feeding mechanism, though perhaps not to veliger locomotion. The food-groove, bounded by its continuous "fences" of the prototroch and metatroch, runs round the margin of both velar lobes (Fig. 2C) and the mouth lies within it on the ventral side. As recently clearly elucidated for the feeding mechanism of trochophores (Strathmann, Jahn and Fonseca, 1972), the opposed beat of the two ciliary bands is responsible for most effective collection of particles. The veliger of *Melampus* is small with a moderately proportioned velum (Fig. 2C) not as large as the velar "wings" of nassariid or naticid veligers, but proportionately larger than those of some littorinids. Functionally it is a relatively continuous swimmer (and therefore food intake is potentially continuous), unlike the veligers of littorinids whose cilia frequently stop for a few seconds. Fed on cultures of *Dunaliella tertidecta* in the laboratory, in conditions far from optimal, we had veligers hatching at 125 μ (shell maximum dimension) regularly reaching 185 μ in eight days (corresponding to rates of 7.5 μ per day, or an increase in mass of about 3.38 times). The field rates must be somewhat higher since the newly hatched veliger at 125 μ reaches its presettlement size of 270 μ in fourteen days (corresponding to 10.4 μ per day).

The largest veliger of *Melampus* that we have found in plankton samples measured 288 μ and our largest cultured veliger was 276 μ (although it was then 20 days from hatching). As already noted, settlement occurs in larvae of between 270 μ and 290 μ. However, our largest cultured veligers had already moved to the postlarval pattern of shell growth (that is, had shells more like Fig. 4A than Fig. 3B). Some observations on such "late" veligers are worth reporting. In life this stage is, in many features of structure and function, intermediate between the free-swimming veliger (Fig. 2C) and the early post-larva (Fig. 4C). It swims reasonably well for short periods, although it looks awkward and as though the relatively small velar lobes are inadequate to lift the new relatively massive visceral mass and shell. It is also capable of crawling, and can move on its well-developed locomotory foot as competently as a spat of 1.2 mm shell-length.

However, the head-foot organization is markedly different from that of the postlarva or spat: the foot is much narrower and more elongate anteriorly (the eyes lie very far back) and posteriorly (where there is a long tail supporting the retained operculum). This intermediate anatomy and behavior may be typical of veligers at settlement although the majority of them will have a shell form closer to that of Figure 3B.

Although our evidence can never be more than circumstantial, we are reasonably sure that the veligers spend about fourteen days in the plankton. In 1970 considerable settlements must have occurred with the spring tides of July 4 and August 2 (corresponding to egg-laying of June 7 and July 6). Sets of spat samples taken in mid-August (see next section) established that, although a fraction of the total settlement can take place below the *Melampus* zone, the bulk of the return takes place into the exact zone occupied by the adults and that the differences in distribution which occur can be explained on mechanical grounds of water flow. For example, denser settlement is found among *Spartina* roots along the edges of drainage channels, where some filtering effect (along with increased contact stimuli to the veligers) must occur as each spring high tide recedes from the marshes. Other typical sites for denser settlement are the centers of shallow depressions within the *Melampus* zone, where again concentration of veligers could occur on a falling tide.

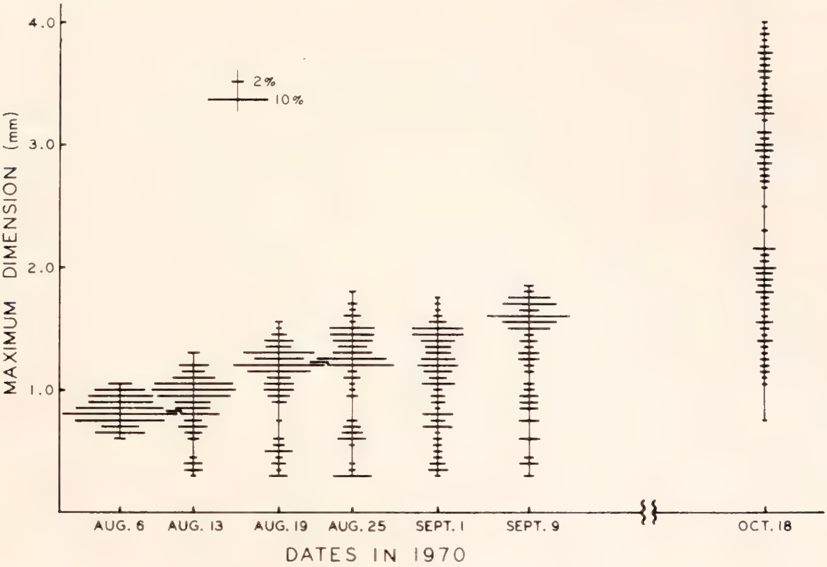

FIGURE 9. The growth of spat stages of *Melampus*. Sets of histograms show size distributions in successive samples of spat from Little Sippewisset in late summer, 1970. Sampling is probably slightly biased against spat of under 0.6 mm maximum shell dimension. Sample numbers ranged from 47 (Aug. 6) to 149 (Aug. 19), but the histograms indicate the percentage of the sample in each class interval to facilitate comparison.

We know that growth of the postlarva immediately after settlement is slow and limited (see Fig. 4 and Section III), and that the remnants of velar lobes and the operculum are lost very quickly. The postlarva shows cryptic responses in marked avoidance of light and air currents at this time. Circumstantial evidence (from the dates and growth of early spat collections) suggests that there is a further slowing of growth rates in the postlarva for the 10 to 14 days preceding metamorphosis. The first true spat (postmetamorphic as in Fig. 5A and B) are around 320 μ in maximum dimension with only a small gain from the early postlarva (Fig. 4C at 290 μ maximum dimension). Timings for settlement and for the postmetamorphic appearance of true spat are also shown diagrammatically in Figure 10 for a three-cycle period.

Spat ecology. It is obvious that direct collection of early spat from the field is almost impossible although, on a number of occasions, "long-arm" mounted dissecting microscopes and suitable illuminators were taken into the field for direct observations of the soil surface in the high salt marsh. In our regular sampling for spat, we cut discs of 9.6 cm diameter from the appropriate levels of the habitat, taking about 3.5 cm depth of soil but shearing off the grasses at less than 1 cm above the soil surface. These discs could be taken to the laboratory, the soil surface between the stubble examined under a low-power dissecting microscope, and all spat picked off using a fine camel-hair brush. Initially, various methods of applying heat and of watering were used to bring spat to the surface, but our standard method involved repeated scanning at laboratory temperatures, relying on a high intensity spotlight to detect spat moving in the tangled algal mat at the soil surface. After completion of sampling, the turf discs were returned to their original positions in the field.

Successive size distributions for spat samples are shown in Figure 9. The youngest postmetamorphic spat are about 320 μ in maximum dimension and, although there is some recruitment to our samples at this level (August 13 through September 9), our techniques may be slightly biased against spat of under 600 μ. However, it is almost certain that the size distribution of spat at August 6, 1970 (Fig. 9) is made up almost entirely of the first of the two major settlements of the summer, that of July 4. Over the 33 days since settlement, these spat have grown from the modal size of 280 μ at settlement to the postmetamorphic size of about 320 μ in some 14–15 days and then to a modal spat size of 800 μ in the following 18–19 days. As can be seen from the figure, the rate of spat growth remains high: a further five weeks of growth taking the median members of this particular cohort to a size of 1.6 mm. The other major settlement of the summer, that of August 2, is represented in the size distributions from mid-August onwards but no clear picture either of two or of four distinct cohorts emerges in the samples of September or October. The cohorts are blurred partly by the other smaller but synchronized settlements of June 21 and July 18, and by any later settlements corresponding to the delayed hatchings of August 2 and August 16 (that is settlements which might yield spat sample recruitment around August 30 and September 12). There is some evidence that the spat derived from later settlements grow more slowly, which could decrease survivorship in later cohorts (see Discussion below). As already noted (Section III), the first four weeks of growth after settlement can yield spat of mean tissue dry weight around 11.3 μg (at a shell length of 675 μ), a hundred times the tissue weight of the newly hatched veliger. For the earliest cohort discussed above (Fig. 9) the median size of 1.6 mm reached by September 9 (that is, about nine weeks after settlement) would correspond to a mean tissue dry weight around 185 μg, a further 16-fold increase in real biomass terms. Thus, for that cohort of veligers hatched at the optimal time, biomass growth can extend through more than three orders of magnitude in the first eleven weeks of life. This is a little faster than that documented in our preliminary note (Russell Hunter and Apley, 1966). Apley (1970) provides detailed population statistics for subadult and adult growth, and from his figures modal tissue dry-weights can be derived of 1.9 mg (at 3.5 mm shell length) after one year of life, and of 10.3 mg (at 7.5 mm shell length) after two years. Russell Hunter and Apley (1966) quote a tissue dry-weight of 81 mg (at 10.1 mm shell length), which would certainly be a snail in its third year. Since individual snails can reach shell lengths of 12.3 mm, life-span growth in real biomass terms, such as tissue dry-weight (or total organic carbon, see Apley, 1967, 1970; Apley *et al.*, 1967; Russell Hunter and Apley, 1966) can certainly encompass six orders of magnitude in 3–4 years. Comparison of the October spat size distribution of Figure 9 with the first year's growth data of Apley (1967, 1970) suggests that the smaller spat (that is, the later-hatched cohorts) are somewhat less likely to survive their first winter. There could be a minimum size at November for survival to the following spring. If this is so, then the termination of the overall reproductive period in mid-July could have adaptive significance (*e.g.*, August spawning could not produce winter-viable spat) as well as reflecting bioenergetic depletion (see Results section I above).

TABLE IV

Summer spring tides which corresponded to semilunar cycles of aggregation, copulation and egg-laying in populations of Melampus *at Little Sippewisset* (○ full moon, ● new moon)

1965	● May 30	○ June 14	● June 29	○ July 13
1966		○ June 3	● June 18	○ July 2
1967		● June 8	○ June 22	● July 7
1968	● May 27	○ June 10	● June 25	
1970	○ May 21	● June 4	○ June 19	● July 3

Annual aspects of synchrony. Over all summers covered by our observations at Little Sippewisset (1965, 1966, 1967, 1968, 1970) the earliest natural egg-laying was on May 23, and the latest on July 13. At this latitude therefore the total reproductive period could extend through a maximum of 51 days. Whether this annual reproductive period encompasses either three or four cycles of copulation and egg-laying would seem to depend on the incidence of spring tides in that particular year, and on an interaction of such *potential times of egg-laying* with the limits set to total egg-production by the sequential bioenergetic depletion. The spring tides corresponding to cycles of copulation and egg-laying for the five years of observations are set out in Table IV. It may be significant that not all cycles of egg-laying are of equal intensity. In both "four-cycle" years, 1965 and 1970, the second and fourth cycles involved massive oviposition, with the first and third being slighter. On these bases, a predictive hypothesis might run as follows. (1) No spring tide before May 21 can support a breeding cycle (on grounds of the latent period after day-length and temperature control). (2) A reproductive period of four breeding cycles can occur only if: (a) the first cycle corresponds to spring tides *before* June 1, and (b) at least one of the first three cycles does not involve massive egg-laying. This hypothesis would accommodate the case of 1968 as being one where the spring tides around July 10 constituted a potential egg-laying cycle, but depletion had already occurred with the third actual egg-laying cycle around June 25.

As in many other biological situations involving synchronization with seasonal changes, the environmental signals utilized by our populations of *Melampus* to control the timing of their reproductive period are not necessarily related to the selective pressures originally responsible for the evolution of that synchronization. The onset of the period is based on the sensory inputs of appropriate day-length and of temperature. The ending of reproduction must involve detection of an environmental cue, as well as the immediate effects of gonad depletion. We have no direct evidence on this, although changing day-length is a more likely factor than temperature in mid-July. Obviously, the adaptive significance of terminating reproduction in mid-July (to ensure the settlement and growth of winter-viable spat) need not be so important at lower latitudes. Earlier reports on reproduction in *Melampus* (Hausman, 1936; Holle and Dineen, 1957; Morrison, 1958) seem to have been based on isolated observations of single breeding cycles, with no appreciation of the semilunar rhythm of reproduction. Morrison (1958) briefly discussed the "problem" of an apparent progression of egg-laying dates from north to south. A progressively *later* onset of reproduction within species of marine littoral invertebrates as one moves to populations in higher latitudes is much more usual (Runnström, 1928; Spärck, 1933; Thorson, 1936, 1946; Hutchins, 1947; Jenner, 1956). Apley (1970) records three breeding cycles with semilunar periodicity in late August and September for a population of *Melampus bidentatus* at Fort Macon, North Carolina. Fort Macon is at 34° 43′ N, compared to 41° 35′ N for Little Sippewisset. The most likely explanation involves the existence of different physiological races of *Melampus* at these different latitudes. It is of interest that Sastry (1970) has postulated similar racial differences between two populations of the bay scallop, *Aequipecten irradians*, in Nantucket Sound, Massachusetts and off Beaufort, North Carolina, this being another of the "unusual" cases where the population at the higher latitude spawns earlier in the year. In the case of *Melampus*, we can now postulate that the evolution of such races may have involved the absence of selection pressures for winter-viable spat in the Fort Macon population where winter temperatures in the salt marshes rarely fall below 5° C. In addition, the best conditions for planktonic veligers off Fort Macon may occur later in the summer, since Williams and Murdoch (1966) have reported peak primary productivity during the summer (rather than spring) months for that sea area.

DISCUSSION

Two aspects of the early life-history of *Melampus* merit further discussion: first, the significance of growth and survivorship problems resulting from the relatively small egg, including the mantle metamorphosis after settlement, and secondly the overall significance of semilunar synchrony.

Among molluscs, as among other marine invertebrates, there are cases of pairs of relatively closely related species differing markedly in their specific egg-sizes (and thus differing inversely in their numerical fecundity). The evolution of larger eggs has involved a balance of selection pressures: small eggs confer advantages in species distribution, and perhaps also in any species' capacity for genetic change; while large eggs confer advantages in survivorship resulting from suppression of free larval stages, and also (as we have suggested earlier, Russell Hunter and Apley, 1966) in reduction of the temporal extent of immature growth. As documented in this paper, in *Melampus* biomass growth in terms of organic carbon or ash-free dry-weight extends through two orders of magnitude (1×10^2) during veliger and early spat (under $675\ \mu$) life, through over three orders (actually 1.6×10^3) during the first eleven weeks of life, and through just over six orders ($> 1 \times 10^6$) in the entire life-span. In contrast, our studies show a variety of "higher" freshwater pulmonates hatching from relatively large eggs (5–40 μg organic carbon), and showing a biomass growth of only two to three orders of magnitude (about 5×10^2) during their life-span [*Physa heterostropha* (Russell Hunter and Apley, 1966); *Ferrissia rivularis* (Burky, 1971); *Lymnaea palustris* (Hunter, 1972); *Laevapex fuscus* (McMahon, 1972); and *Ancylus fluviatilus* (Russell-Hunter and Burky, in preparation)]. The eggs of most land pulmonates are proportionately even larger, and the extent of biomass growth from egg to adult still further reduced. Discussions of the evolution of larger eggs in non-marine environments usually emphasize the "need to suppress the free larval stages." Our data on the orders of magnitude involved in real measures of growth, such as organic carbon, suggest that selection pressures to reduce the temporal extent of immature growth have also influenced the evolution of larger eggs. The "start in life" of being born large is important in environments with marked seasonal changes and, in the various species of freshwater pulmonates noted above, has made possible both strict annual and certain bivoltine patterns of life-cycle (see also Russell-Hunter, 1964, 1970).

In most molluscs, as in certain other invertebrates, there is a close inverse relationship between egg-size and numerical fecundity which reflects the maximum possible production of egg-biomass by the parent. Most benthic marine invertebrates with small eggs yielding planktonic larvae produce egg numbers in the range 10^3 to 10^5 per female per breeding season (Thorson, 1950; see also Scheltema, 1971). The numerical fecundity of the hemaphroditic *Melampus* lies neatly within this range at 33,150 eggs per snail per year. Numerical fecundities for the large-egged freshwater pulmonates listed above (all also hermaphroditic) lie in the range 8–800 eggs per snail per year. Retention of the "primitive" pattern of small eggs and high numerical fecundity in *Melampus* must reflect long-term adaptive advantages. These include the enhanced species distribution which results from a planktonic period in the life-cycle since the habitat provided by salt marshes is geographically discontinuous. Another factor which may be of long-term adaptive significance is the increased capacity for genetic change conferred by a higher numerical fecundity and a longer elapsed time between hatching and maturity (the period of effective natural selection). Obviously the probability of survival to maturity of any individual egg in a species with near-Darwinian populations (nearly constant population numbers from generation to generation) is inversely proportional to the average life-time numerical fecundity. In reporting earlier molluscan studies, Russell Hunter (1957) had used an "annual ratio of selection in stable populations" (page 69) to express this relationship. If the life-time fecundity of *Melampus* is prorated at about 55,000 (applying the age-structure and survival of natural populations at Little Sippewisset to our mean laboratory fecundity of 33,150 per snail per reproductive season), then this can be contrasted with life-time fecundities in the range 8–70 for different populations of certain ancylid limpets (*Ancylus*, *Ferrissia* and *Laevapex*: Russell Hunter, 1953a; Burky, 1971; McMahon, 1972). Ancylid limpets can be regarded as among the most highly specialized of freshwater pulmonates, and the low level of fecundity is probably correlated with this. If the "primitive" pattern of reproduction in *Melampus* with high numerical fecundity confers certain long-term adaptive advantages, some of the short-term disadvantages in a more stochastic pattern of survivorship must be countered to some extent by the multiplication of separate egg-laying cycles within each reproductive period.

Again as a result of the relatively small egg and the need for active food intake and growth as a veliger, *Melampus* shows a profound trophic shift at settlement—a functional metamorphosis if not a change in form. Filter-feeding by velar cilia must be replaced within a few hours by the processes of active radular grazing which will continue throughout adult life. The more obvious metamorphosis of shell which occurs a little later (some days after settlement) in *Melampus* is one of the mantle-edge and of the secreted shell shape which it generates (see Section III, above) and this metamorphosis does *not* involve any reversal of asymmetric internal organs such as the kidney and the auricle of the heart. Throughout the embryonic, larval and spat stages of *Melampus*, the internal organs are in their adult *dextral* arrangement. Thus the terms sinistral (for the late veliger and post-larval shells) and dextral (for the shells of metamorphosed spat and of adults) were used above only in their simpler descriptive sense as regards the apparent direction of shell-coiling but *not* in the more specific sense used in gastropodan comparative anatomy as regards a mirror-image asymmetry of all the unpaired internal organs. If the terms sinistral and dextral are restricted to this morphological usage for cases of entire reversal of symmetry or *situs inversus* of all organ systems (which in turn reflect mirror-reversal of the planes of spiral cleavage in the egg), then we must describe the shells of the later veliger and postlarva in *Melampus* (Figs. 3AB and 4AB) as hyperstrophic, and the shells of metamorphosed spat and of adults (Figs. 6ABC and 7ABC) as orthostropic. An early elucidation of hyperstrophy (or those cases of apparently sinistrally coiled shells enclosing snails with dextrally arranged internal anatomy) occurs in Simroth (1896–1907; see also Pelseneer, 1891, 1892, 1906), and more recent accounts include those of Brookes Knight (1952), Cox (1960), and Fretter and Graham (1962). Metamorphosis of the mantle and shell in *Melampus* from a hyperstrophic to an orthostrophic condition (with dextrally coiled shell enclosing dextral anatomy) is paralleled in a number of other snails with planktonic larvae (Fretter and Graham, 1962; Robertson, 1963; Robertson and Merrill, 1963). Among these are many species of those Pyramidellidae (a family now regarded as opisthobranch) which have planktonic veligers. Apparently those pyramidellids in which the planktonic veliger is suppressed do not show any heterostrophy or shell metamorphosis. As Fretter and Graham (1962) have discussed, the pyramidellids have many features which could be interpreted as those of a "stem group" transitional between opisthobranchs and prosobranchs. That the nature of shell metamorphosis is *Melampus* should be similar is striking, since Morton (1955a, 1955b) has pointed out the many archetypic features of nervous, excretory and reproductive anatomy in ellobiid pulmonates which appear to link the higher pulmonates through them to the same prosobranch stock (the Rissoacea-Cerithiacea group of monotocardians) from which the opisthobranchs may have evolved (see also Fretter and Graham, 1962).

No matter the significance of such attempted phyletic correlations (and extensive dialectics on the significance of torsion are involved here), and despite the complex terminology of shell-coiling, a simple explanation in adaptive-functional terms can be put forward for most, perhaps all, of the growth changes of the mantle-shell in *Melampus*, including the metamorphosis. The explanation, mechanistic and Ockhamistic, must lie in the changing needs for protective containment of the changing proportions of the head-foot and visceral mass. The genetic controls, which create the growth gradients of the shell aperture by generating the differential rates of cell division in the cells of the mantle edge, have been adapted to switch appropriately to best enclose the changing asymmetries of the internal parts. Within the egg-shell, even after torsion, growth of the mantle-edge is bilaterally symmetric and, if continued, would generate a planospiral shell. During planktonic life, the mantle of the veliger produces a shell with a rapidly enlarging aperture and relatively disjunct or "open" turbinate coiling. For this period, shell growth shows some allometry. As already noted (Observations, Section III), both these shell features and the retention of an operculum can be explained by the need to accommodate the large velar lobes. After settlement, loss of the velum is accompanied by isometric and slower enlargement of the shell aperture in the postlarva. If this growth continued, we should have a hyperstrophic shell through the adult, but *it does not* and we have that profound metamorphosis of the mantle which leads to the generation of the orthostrophic (true dextral) coiling of spat and adult. With moderately turbinate shell growth, adult hyperstrophy would bring the spire anteriorly (or at least anteriolaterally on the left side) and, despite torsion, would result in similar disadvantages (in the mechanics of locomotion and of sanitation) to those hypothesized for exogastric as opposed to endogastric coiling (Pelseneer, 1906; Naef, 1911; Yonge, 1947; Ghiselin, 1966). It seems highly significant that mantle-shell metamorphosis in *Melampus* occurs only a few days after settlement and the shift from a swimming to a crawling habit. As now seems to hold for certain peculiarities earlier claimed in the shell growth of ancylid limpets (Russell Hunter, 1953a; Russell-Hunter and Nickerson, in preparation, on biometrics of *Ferrissia*), the simplest adaptive interpretation in terms first of the necessary containment of head-foot with visceral mass, and secondly of locomotory efficiency, may best account for all the shifts of shell-mantle growth in *Melampus*.

The unique combination of primitive and of specialized features, which characterize different aspects of the physiology of *Melampus*, creates the need for the strict synchronization of such processes as egg-laying, hatching and settlement. Although amphibious snails, in their respiration they are relatively specialized, breathing air through pneumostome and lung as in the true land snails. Adult specimens of *Melampus* can be drowned by prolonged submergence. It appears that *Melampus* is unable to use an exposed gas bubble as a "physical gill" for sustained diving, as can be done in certain pulmonate genera (including *Lymnaea*, *Physa* and *Planorbarius*) which have become "readapted" for aquatic life in fresh waters (see Russell Hunter, 1953b; Henderson, 1963; Russell Hunter, 1964). Given this inability to live submerged, and the general topography of salt marshes, it is clear why adults of *Melampus* do *not* migrate down to lower zones of the littoral for reproduction as do some sympatric arthropods. On the other hand, the relatively primitive pattern of reproduction in *Melampus* involves the spawning of large numbers of small eggs which yield planktonic larvae on hatching.

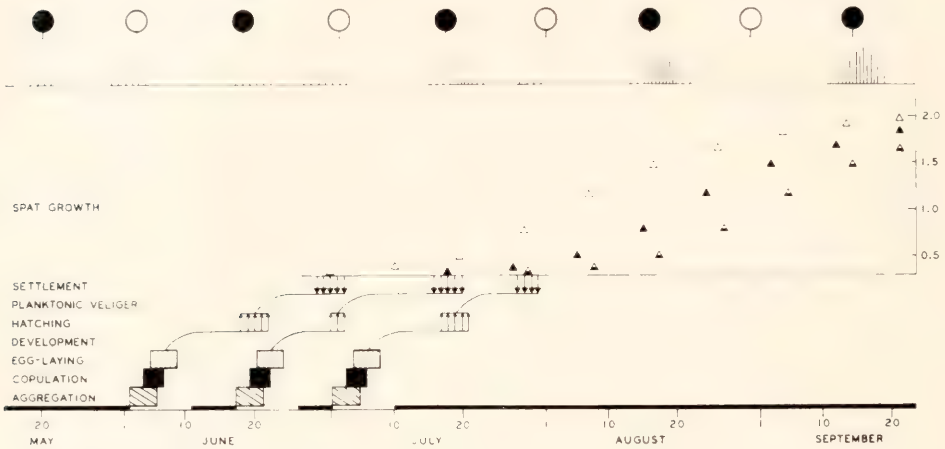

FIGURE 10. Semilunar periodicity in reproduction and early life-cycle of *Melampus* at Little Sippewisset. The diagram is based on the actual timings and observations of the "three-cycle" reproductive period of summer 1966, except for the spat growth which is interpolated from the more extensive spat data of 1970. At the top of the figure lunar phases are shown conventionally, with below them a record of those high tides which exceeded a vertical height of 4.4 feet (the height of the top of the *Melampus* zone in Fig. 8). At the bottom of the figure, the thicker portions of the base-line indicate times when the population of *Melampus* was dispersed. Three cycles of aggregation, copulation and egg-laying occur at semilunar intervals. Hatching and settlement also show semilunar synchronization. Three kinds of triangles are used to distinguish successive modal sizes for the three cohorts of spat which result from the three cycles of egg-laying in this reproductive period, and the vertical scale at the right indicates the maximum shell dimension of those spat in millimeters. For further explanation and discussion, see text.

As specialized lung-snails, populations of *Melampus* live in the upper 12% of the intertidal zone. Since they retain the reproductive pattern of primitive marine snails, they can spawn only during the 2.3% to 4% of each semilunar tidal cycle when their habitat is bathed by seawater. Hatching of planktonic veligers and resettlement must also be similarly synchronized. The acuity of these problems of temporal "fitting" in *Melampus*, as compared with other intertidal animals, results from those two divergent sets of physiological features. The considerable evolutionary interest of the ecology and physiology of ellobiid snails like *Melampus* stems from this combination of the primitive and the specialized. Contemporary ellobiids possibly live in similar habitats to those of the Jurassic ancestors of the successful land-snails of today, and undoubtedly they face similar physiological problems. The retention of archetypic reproduction in *Melampus* over this long evolutionary period implies not only successful, but also adaptable, methods of seasonal and semilunar synchronization. The possible occurrence of physiological races of *Melampus* adapted to different latitudes is significant in relation to this apparent evolutionary conservatism. Even more striking in this regard is the contemporary existence in the controls of semilunar synchronization both of obligate and of facultative processes—a mixture which undoubtedly is of adaptive significance in conferring a flexibility of resynchronization and thus a long-term capacity to adjust to varying tidal circumstances.

The first necessary synchronization, that of aggregation, copulation and egg-laying with the days of spring tides, is an obligate process. The appropriate behavioral shifts and the internal changes in the reproductive tract preparatory to copulation and egg-laying occur in laboratory stocks at times of full and of new moon, even when these stocks have not experienced the rhythms of tidal submergence for ten semilunar cycles. It is not unlikely that the day-length and other controls of the onset of the annual reproductive period are "paced" to some extent by the same "biological clock," or perception of more subtle semilunar geophysical changes, as dictates the rhythm of copulation and egg-laying. Whatever the mechanism, egg-laying occurs with a semilunar synchrony despite the absence of tidal bathing.

The second synchronization, that of hatching, is different. Conceivably, it too could be obligate and depend on a rigorous developmental timetable following synchronous fertilization, egg-laying and first cleavage, although the necessary temperature independence is somewhat unlikely. In fact, as reported above, the synchronous hatching process is facultative and depends on the incidence of tidal submergence. As confirmed by our laboratory experiments, hatching of *Melampus* in the field must usually be brought about from egg-masses of age over 10 days (and under 21 days) by a sequence of about four tidal floodings. The adaptational significance of the innate flexibility in the time of hatching is that, by this extension of survival, it can provide for the successful overlap of cohorts from different semilunar cycles of egg-laying. On the other hand, although hatching is a facultative process, a resynchronization of the early life-history in *Melampus* is achieved by the dependence on a sequence of floodings (occurring with a semilunar periodicity in the field), and this resynchronization is undoubtedly of great significance to the future synchronous resettlement of veligers into the appropriate salt-marsh habitat at a later cycle of spring high tides.

Settlement is an important problem for all intertidal benthic invertebrates with planktonic larval stages. In the majority of such forms there are physiological and behavioral adaptations which effectively increase the chances of resettlement into the appropriate zonal habitat. Aspects of the interaction of temporal controls and the immediate environmental stimuli on settlement of planktonic larvae have been ably summarized by Wilson (1948, 1952), who also provides one of the best expositions of the important ability of *some* larvae to delay settlement long after they have reached an appropriate stage of development, if in the meantime they have not encountered the specific environmental conditions appropriate to their settlement. Recently Scheltema (1971) has discussed the importance of zoögeographical distribution of this capacity for prolongation of larval life in some prosobranch gastropods. Veligers of *Melampus* seem to have some capacity to delay settlement —at least for a few days. The problems of resettlement into the appropriate intertidal zone are especially acute for animals of the supralittoral fringe like *Melampus*, particularly when there can be only a limited possibility of upward migration by spat after settlement. We have no evidence of any species-specific chemoreception (either repellant, see Wilson, 1952; or attractant, see Knight-Jones, 1953) being important in the settlement of *Melampus*. Although a few larvae settle just *below* the adult *Melampus* zone, the great bulk of settlement occurs into that exact zone. Thus it occurs in the field only at spring high tides. Although some aspects of this remain obscure, settlement as a process undoubtedly involves only veligers of a suitable age and size, and may involve a definite level of local mechanical stimuli for completion.

Semilunar synchronization of the events of reproduction and of early life-cycle is clearly necessary in contemporary populations of *Melampus*. A long evolutionary history of the capacity for this rhythm is suggested by the retention of an archetypic pattern of reproduction and larval life. In turn, this could have been made possible by the flexibility of resynchronization conferred by the use of a combination of obligate and facultative processes to achieve the required synchrony at each stage.

We wish to thank Jay Shiro Tashiro for his skilled help in the field and the laboratory during several summers, and we are also grateful to John L. Banner III and Sandra E. Belanger for their assistance in the final preparation of this paper.

SUMMARY

1. The salt-marsh pulmonate snail, *Melampus bidentatus,* is placed in the Ellobiidae which family encompasses the most primitive of living Pulmonata and is regarded as not far removed from the ancestral stem-group of both modern land snails and freshwater pulmonates. Inhabiting the higher levels of salt marshes, *Melampus* is "amphibious": although an air-breather with a gill-less vascularized mantle-cavity functioning as a lung, it retains an archetypic pattern of reproduction with small eggs and a free-swimming veliger larva.

2. Field and laboratory studies over several years (based on natural populations at Little Sippewisset, Cape Cod, Massachusetts) have shown that egg-laying, hatching, and larval settlement are each confined to cycles of about four days in phase with the spring high tides. Adaptively such semilunar synchronies ensure that these processes occur only during the 2.3% to 4% of each month when the *Melampus* habitat in the upper 12% of the intertidal zone is bathed by seawater.

3. The annual reproductive period extends from late May or early June through early July, with either three or four cycles of egg-laying occurring at two-week intervals in phase with the tides of new and of full moon. Synchrony of egg-laying (and of the patterned aggregation and copulation which precede it) is obligate.

Stocks of *Melampus* brought into the laboratory in spring will maintain the same semilunar rhythm of reproductive behavior during the summer period.

4. Eggs are small (about 109 ng organic carbon) and are laid in gelatinous egg-masses averaging 850 eggs. Mean numerical fecundity is 33,150 eggs per snail per year. For most freshwater pulmonates fecundity would lie in the range 8–800 eggs per snail per year. At 18° C, development to a well-differentiated and active veliger within the egg-shell takes 11 days.

5. Hatching shows semilunar synchrony in the field: enormous numbers of newly hatched veligers can be collected on the flood of appropriate spring tides. A series of experiments with laboratory-laid egg-masses showed that eclosion normally occurs in response to a sequence of about 4 tidal floodings in under 50 hours. Hatching can occur from egg-masses from 10 to 24 days after laying. Being facultative, the process allows better survival and overlap of cohorts but also reestablishes the synchronization with spring tides.

6. Veligers feed actively and grow from shell length 127 μ to 280 μ during their time in the plankton, deduced to be 14 ± 2 days. The bulk of the settlement is into the exact vertical zone occupied by adult *Melampus*.

7. A period as a crawling, radula-feeding postlarva (after loss of velar lobes and operculum) is followed by an abrupt metamorphosis of the mantle and shell. Postmetamorphic spat grow rapidly. In terms of organic carbon or ash-free dry weight, growth extends through two orders of magnitude during veliger and early spat life, through more than three during the first eleven weeks, and six in the entire 3–4 year life-span. In contrast, similar biomass growth measures in freshwater pulmonates involve only two to three orders in their life-span.

8. In *Melampus*, the shells of late veligers and of post-larvae show sinistral coiling, and those of metamorphosed spat and of adults dextral coiling. There is a metamorphosis of mantle and shell alone; throughout development, larval and spat stages, the internal organs are in their adult dextral arrangement. Such a metamorphosis from a hyperstrophic shell condition to an orthostrophic one is known to occur in the ectocommensal opisthobranch family Pyramidellidae and in certain other snails with planktonic larvae. The present study provides the first description of the succession of shell stages and metamorphosis for any pulmonate.

9. In conclusion, the small eggs, the mantle-shell metamorphosis, and the semilunar synchrony are discussed in their evolutionary setting. "Primitive" reproduction with small eggs, as retained in *Melampus*, confers advantages in dispersal and genetic potential. Evolution of larger eggs, as in the freshwater pulmonates, may have involved selection pressures to reduce the temporal extent of immature growth in seasonally variable environments. Mantle-shell changes in *Melampus*, including the metamorphosis, can be interpreted simply in terms of the changing needs for protective containment at different stages in the life-cycle. Semilunar synchrony of reproductive and of larval stages has evolved in response to the concursion of specialized aerial respiration and the primitive pattern of spawning large numbers of small eggs. The combination of both obligate and facultative processes in producing these synchronies is thought to be significant in relation to the long evolutionary history which can be hypothesized for these semilunar rhythms.

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ADAPTATIONS TO ENVIRONMENTAL OXYGEN LEVELS IN INFAUNAL AND EPIFAUNAL SEA ANEMONES

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Numerous investigators have shown correlations between various physiological properties of aquatic organisms and the characteristic levels of oxygen in which the animals are found. Older studies on survival time under low oxygen conditions generally indicate that, within closely related groups, burrowing species are more resistant to oxygen deprivation than epifaunal forms (Packard, 1905). Similarly, animals living in fast-moving streams are less resistant than those living in relatively unmixed pond water (Fox, Simmonds and Washbourn, 1935; Bovbjerg, 1952; Walshe, 1948). Walshe (1948) also showed that, among the chironomid larvae, resistance to oxygen lack is better correlated with ecological distribution than with phylogeny.

Numerous studies support the very plausible notion that animals from low oxygen environments have a lower rate of oxygen consumption than their counterparts from high oxygen environments when compared at the same oxygen concentrations. Examples are two species of *Balanus* (Prasada Rao and Ganapati, 1968); epifaunal and infaunal tropical echinoids (Lewis, 1968); oxygen minimum layer mysids (Childress, 1971); stream and pond insect larvae (Fox, Simmonds and Washbourn, 1935), crustaceans (Fox and Simmonds, 1933) and leeches (Mann, 1956); and maldanid polychaetes (Mangum, 1963, 1964a).

Perhaps the most elusive physiological correlate of environmental oxygen level is the degree to which oxygen consumption rate is maintained constant over a range of ambient oxygen concentrations. It is clear that in many aquatic invertebrates respiratory regulation within a species is not entirely constant. It varies with temperature (Thomas, 1954; Wiens and Armitage, 1961), weight (Helff, 1928), molt cycle (Thompson and Pritchard, 1969) and previous activity levels (Nimura and Inoue, 1969).

Relatively few investigators have examined the metabolic response of aquatic animals to wide ranges of oxygen concentration following periods of oxygen deprivation. Instead, most investigations have characterized the response to anoxia only at oxygen concentrations at or near air saturation. Prosser, Barr, Pinc and Lauer (1957) have shown that goldfish respond to chronic exposure to low oxygen conditions by a reduction of standard metabolism and a shift of critical pO_2 to lower oxygen partial pressures, accompanied by increased hemoglobin concentrations and red blood cell counts. After oxygen lack, oxygen consumption rates in the mud snail *Nassarius obsoletus* increase (Kushins and Mangum, 1971), but the response of oxygen sensitivity is variable. While oxygen sensitivity may either decrease or show no change, the most common response is to increase. As yet, no clear picture has emerged of the response of marine invertebrates to wide ranges of oxygen concentration following chronic lack of oxygen.

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Many comparative studies of adaptation to environmental oxygen level have been concerned only with selected aspects of the animals' responses to oxygen concentration. The present investigation is based on the premise that an understanding of the adaptations of animals to the prevailing levels of oxygen in their respective habitats requires consideration of oxygen distribution in the environment and some knowledge of the manner in which oxygen enters the animal, as well as the kinetics of its consumption in relation to stress situations. Thus, the present study has centered around various morphological, behavioral, and physiological aspects of adaptation to low oxygen in two species of sea anemones that are distinct ecologically with reference to the oxygen characteristics of their respective environments.

METHODS AND MATERIALS

Habitat

Metridium senile (L.) is a characteristically epifaunal species attached to rocks and pilings in semi-protected areas of the New England coast and south to New Jersey. *Haloclava producta* (Stimpson) is a burrowing form living in medium grain sand; those animals used in this study were collected from Waquoit Bay, Massachusetts. Both species were provided by the Supply Department, Marine Biological Laboratory.

A sediment sample of 570 g (dry weight) containing 3 *H. producta* was taken in July 1971 at Waquoit Bay. The sample was rinsed, soaked in 1 N NaOH, rinsed again, and dried at 60° C for 24 hours. The dried sample was then sieved through a standard series (Mangum, 1964b) and each fraction weighed.

In the laboratory the burrow shape and orientation of *H. producta* was observed in a sandwich of sand held between two glass plates. Two to four animals were placed in the apparatus at the same time. They were allowed to burrow and were subsequently observed over a period of several weeks.

Behavior

Two facets of behavior in *Haloclava producta* were examined: (1) spontaneous activity of the column of intact animals and (2) irrigation of artificial burrows. Spontaneous activity of the column of intact animals was recorded by allowing each animal to adhere to the inside wall of a small piece of glass tubing and then hooking the free tentacular crown to a kymograph lever by the method of Batham and Pantin (1950a).

Irrigation activity of *H. producta* in straight glass tubes was kymographically recorded in the apparatus described previously (Mangum, 1964a). In addition a geometrical estimate of irrigation rate was made by calculating the volume of water pinched off in each peristaltic irrigation wave of an animal in a glass tube and multiplying this value by the frequency of the waves.

Distribution of oxygen

Field levels of oxygen concentration were determined in Waquoit Bay. Interstitial water samples of 25 ml were taken from depths of 5 cm and 10 cm in the sediment. The samples were drawn anaerobically into a 20 ml syringe fitted with a #13 gauge needle to prevent clogging with sand. A maximum of 0.5 ml sand was included in the sample. Immediately after the sample was taken, the plunger of the syringe was replaced with a calibrated galvanic oxygen probe (Precision Scientific Co.), leaving no air space, and the water was mixed by inverting the syringe and probe several times while holding the tip closed. The movement of introduced sand grains provided mixing and obliterated oxygen gradients around the probe head. Two samples were taken at each depth and several additional measurements were made on the oxygen content of the water overlying the sand. The results were similar to *in situ* measurements obtained by rotating the probe in the water or sand, although we were less confident in this case of adequate mixing.

Measurements of oxygen concentration in the running seawater system in the laboratory were made with a polarographic electrode (Yellow Springs Instrument Co. model 5420) and with the pO_2 module of a Radiometer Blood Gas apparatus (BMS1 equipped with acid-base analyzer PHM1). The oxygen content of glass tubes occupied by *Haloclava producta* was determined by removing 50 μl of water from each tube ($N = 15$) and measuring its pO_2 with the Radiometer Blood Gas apparatus.

The oxygen level in the gastrovascular fluid of *Metridium senile* equilibrated to running seawater was determined with the Radiometer apparatus on samples anaerobically removed from each of 10 large (diameter *ca.* 8–12 cm) animals with a 1 ml syringe. The oxygen content of gastrovascular fluid from *H. producta* burrowed in a few cm of sand and held in running seawater was similarly determined, but pooled samples from groups of three of these very small (*ca.* 0.5 cm) animals were used to make each of 2 determinations.

Survival

Survival of animals of the two species under low oxygen conditions was determined by placing them in darkened chambers containing seawater flushed with nitrogen. The initial concentration of oxygen in the chamber was 0.5–0.7 ppm. After introduction of the animals the chamber was sealed with a rubber or ground glass airtight stopper and kept at room temperature (22–24° C) for the desired dosage time. At the end of this period the animals were returned to running seawater until criteria for either mortality (decay and disintegration) or survival (muscular response to tactile stimulation) were met. Animals that survived were maintained in running seawater for an additional 24–48 hours before being definitively scored. Simultaneous controls were maintained in running seawater at 18–22° C.

Anatomy

Anatomical observations were made on the distribution of ciliated epithelial surfaces, complexity and bulk of internal structures, body wall thicknesses and the distribution of external surface area in both species. Ciliary activity of the column and tentacles of living animals, and of the actinopharynx and mesenterial filaments of freshly dissected animals, was observed with carmine and india ink suspensions. The arrangement and number of mesenteries and body wall thicknesses were determined on preserved and sectioned material. Animals of comparable column diameter were anesthetized with $MgSO_4$, fixed in Bouin's solution, embedded in paraffin, sectioned at the level of the actinopharynx, and stained with hematoxylin and eosin. In calculating external surface area the body wall and each individual tentacle were approximated by cylinders.

Metabolic response to diminishing concentrations of oxygen

In view of the findings of numerous investigators (Helff, 1928; Thomas, 1954; Van Dam, 1954; and Wiens and Armitage, 1961) that the magnitude of change of oxygen consumption rate with reduced oxygen concentrations is influenced by environmental factors, the metabolic response curve of *Metridium senile* was determined under a variety of conditions. Ten different conditions were produced by creating nine combinations of acclimation temperature and experimental temperature, and by exposing one group of animals to anoxia for 24 hours before determining metabolic response to diminished oxygen. These conditions and the number of animals in each group are given in Table I.

Individual animals were allowed to set in darkened respiration chambers of 30, 60, 120 or 300 ml volume. After a period of temperature acclimation (at least 3 days) the chambers were placed in a constant temperature water bath and were sealed by the insertion of a Yellow Springs Instrument Co. Model 5420 oxygen probe. The animals were allowed to reduce the oxygen concentration to a low level (at least below 33% air saturation), and the oxygen concentration monitored by a Model 54 meter (YSI) connected to a Model 80 chart recorder (YSI).

Weight specific oxygen consumption rates were calculated for each 15 min interval and analyzed as a function of the median oxygen concentration in the chamber during that interval. Linear and semilogarithmic regression coefficients were calculated for the relationship between oxygen consumption and oxygen concentration in each experiment. The regression coefficient b is an index of the magnitude of the change in oxygen consumption rate as a function of change in oxygen concentration, assuming a time-independent relationship. The use of the coefficient b as a quantitative description of the relationship between oxygen consumption rate and oxygen concentration is valid only if the slope of the response curve is not changed by the rate at which the animal encounters low oxygen conditions. If the slope of the response curve is affected by the rate of change in oxygen concentration, then the change in consumption rate divided by the change in concentration (this quotient being b) is inversely proportional to time.

To evaluate the possible importance of time dependence, each experiment was treated as a first order kinetic reaction and the corresponding rate constant (k) was calculated from the time required to reduce the oxygen concentration by 50% from the formula $k = 0.693/t_{\frac{1}{2}}$ where k has units of hr^{-1}. If time dependence is a factor in the metabolic response, b should be directly proportional to k. To describe the relationship, b was plotted as a function of k. The effect on b of the various environmental conditions of the 10 experimental groups indicated in Table I is considered below.

Metabolic response to prolonged anoxia

Metridium senile and *Haloclava producta* were maintained in the laboratory for at least three days in running seawater at 17–19° C. They were allowed to set individually in darkened respiration chambers of 300 ml volume (*M. senile*) or 25 ml volume (*H. producta*). Each chamber was sealed with an oxygen probe and oxygen depletion was monitored until oxygen consumption reached zero at 17° C (± 0.05° C). The probe was then removed and the chamber resealed with either a rubber or ground glass stopper. After 24 hr the chamber was refilled with air saturated seawater and the depletion of oxygen monitored again until oxygen consumption reached zero.

For each pair of experiments (before and after exposure to 24 hr of anoxia) the consumption rate at each integral value of oxygen concentration (in ppm) was calculated from best fit semilogarithmic regression lines and both the absolute and per cent increases of the post-anoxic measurement over the initial measurement were determined. The statistical significance of these increases was evaluated using Student's *t* test for paired observations. The difference in oxygen concentration at which oxygen consumption reached zero was analyzed for each pair of experiments using the same test. In addition, the time required for each animal to reach a maximum rate of oxygen consumption following introduction into air saturated seawater after 24 hr exposure to anoxia was analyzed as a function of animal weight using regression analysis.

RESULTS

Habitat

Waquoit Bay is a shallow, broad pan of water on the southern shore of Cape Cod, Massachusetts. The results of grain size analysis of *Haloclava producta* occupied sediment taken there indicate that the modal size class for the particles is 500–999 μ (42.61% dry weight) with nearly as large a fraction (41.91%) in the 250–499 μ range. These two size classes dominate the sediments at 5 of 6 other stations along the southern shore of Cape Cod (Mangum, Santos and Rhodes, 1968).

In the laboratory, *H. producta* burrows over a period of several hours to a depth of at least 10–15 cm. The animals attach the physal disc to the bottom of the observation apparatus and maintain the oral disc flush with the surface of the sand. In their burrows the animals are rather vermiform, extending over 10 cm in length and about 0.6 cm in diameter. The burrows, apparently not highly stabilized by secretory products, easily collapse.

If animals are left undisturbed in the glass plate apparatus for several weeks, organic detritus is deposited on the surface of the sand. A small area around each burrow, however, is kept clear of detritus by the feeding activities of the anemones. As in the case of *Phyllactis concinnata* (D. C. Mangum, 1970), nonburrowed *H. producta* do not respond to particulate food.

Behavior

The column of intact *Haloclava producta* shows a rhythmic pattern of contraction and relaxation similar to those already described for *Metridium senile* (Batham and Pantin, 1950a) and *Diadumene leucolena* (Sassaman and Mangum, 1970). The frequency of the pattern is 12 (± 1.3 S.E.) contractions/hr (3.5 hrs recording time).

H. producta also irrigates artificial burrows with cyclic peristaltic waves which originate below the tentacular ring and are propagated aborally. Observations of four different individuals revealed periodicities of 25, 25, 25 and 24 sec between pumps, with the waves traveling at approximately 0.2 cm/sec. Geometrical analysis of an anemone irrigating a glass tube yielded an approximate pumping volume of 148 μl/pump which (with the above periodicity) gives a rate of 21 ml/hr.

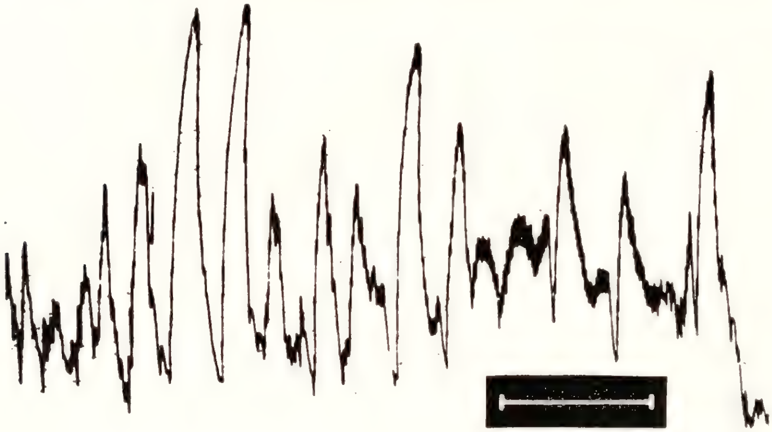


FIGURE 1. Kymographic recording of *Haloclava producta* irrigating an artificial burrow. Scale indicates one hour.

Kymographic recordings of irrigation behavior (Fig. 1) show that irrigation can continue for substantial periods uninterrupted. Indeed, our longest recordings of continued irrigation activity exceed 24 hrs. Quantification of irrigation rate from calibrated kymograph records gives a pump volme of 127 ± 11.8 μl (based on 42 pumps) and a frequency of 71.2 ± 5.0 pumps/hr (based on 5 hr recording time) for an irrigation rate of 9.0 ml/hr. Since a typical 0.5 g animal consumes 15 μl O_2/hr, it must utilize approximately 33% of the 50.5 μl O_2 made available by irrigation of the burrow with air saturated water from above.

Peristaltic waves are not uncommon in anthozoans, having been reported in a pennautlid, *Pteroides griseum* (Brafield and Chapman, 1967), and waves from pedal disc to oral disc are known to aid in body wall cleansing and defecation in *Metridium senile* (Batham and Pantin, 1950b). We are not, however, familiar with any previous report implicating peristaltic waves in burrow irrigation by anthozoans.

Distribution of oxygen

The oxygen content of interstitial water in Waquoit Bay is low. At 5 cm depth in the sand the interstitial pO_2 ranges from 0–5.1 mm Hg, and at 10 cm depth it ranges from 7.5–12.0 mm Hg. These values compare well with those of Brafield (1964), whose study of interstitial oxygen levels in the Scilly Isles included sites of virtually identical sediment composition. Comparable values in similar sediments have also been recorded elsewhere (Eliassen, 1956; Mangum, 1964a; Petersen and Johansen, 1967). Water overlying the sand at the sampling site is over 90% air saturated. Similarly, water in the running seawater system at the Marine Biological Laboratory is virtually air saturated (Fig. 2).

The oxygen content of water in artificial burrows occupied by *Haloclava producta* is equivalent to 128 ± 8 (S.E.) mm Hg. This value does not include an incomprehensible set of measurements on one animal that did not measurably irrigate and in whose tube the pO_2 varied between 46 and 60 mm Hg. The relatively high pO_2 of the artificial burrow water suggests that burrow irrigation in *H. producta* is sufficient to maintain a high effective environmental pO_2, at least during periods of tidal submersion. That these high pO_2's are maintained persistently in artificial burrows reflects the moderately low fraction of oxygen removal from the irrigation stream relative to rates of oxygen utilization in other infaunal animals (Mangum, 1964a, 1970). The results from the single anomalous animal suggest that the absence of burrow irrigation is accompanied by a substantial decrease in burrow oxygen concentration.

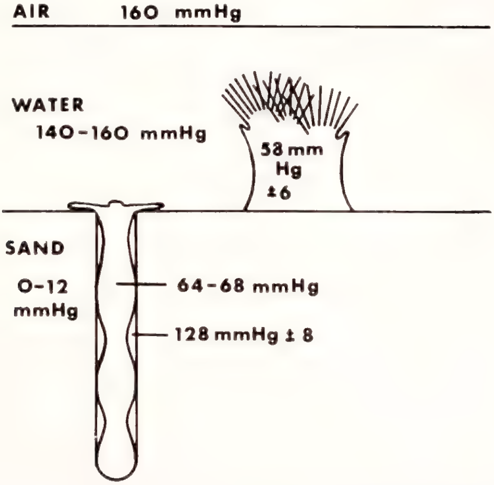


FIGURE 2. Distribution of oxygen in water, sand, burrow and animals. S. E. given for all sample sizes greater than 5, otherwise range.

Internal pO_2's of *H. producta* and *M. senile* equilibrated to running seawater (7.6 ppm) are 64–68 mm Hg and 58 ± 6 mm Hg (= 3.14 and 2.75 ppm), respectively. Brafield and Chapman (1965) have shown that *Pennatula rubra* equilibrated to 6.9 ppm has an internal oxygen concentration of 3.1 ppm and that

Pteroides griseum equilibrated to 8.3 and 6.6 ppm has internal oxygen concentrations of 2.15 and 3.95 ppm, respectively. It is interesting to note that in all four anthozoan species for which concentration differences have been estimated, the internal oxygen concentration falls in the narrow range of 41–47% of the external concentration (based on a mean value for *P. griseum*). The various levels of oxygen at all major loci for *M. senile* and *H. producta* are summarized diagrammatically in Figure 2.

Survival

Metridium senile survives low oxygen conditions uniformly for 96 hours. Dosage times above 120 hours produce uniform mortality. *Haloclava producta* shows no mortality, at least during the first 266 hours; which was the longest dosage attempted. Simultaneous controls show uniform survival.

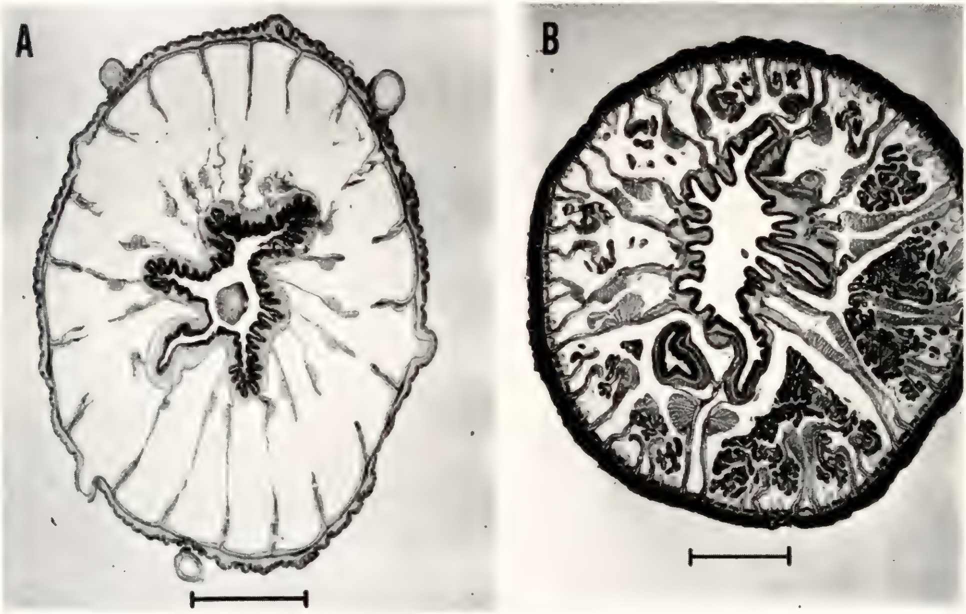


FIGURE 3. (A) Cross section of *Haloclava producta* at the level of the actinopharynx. Scale indicates 1 mm. (B) Cross section of *Metridium senile* at the level of the actinopharynx. Scale indicates 1 mm.

The resistance of *H. producta* to low oxygen is apparently paralleled by a a resistance to internal hydrogen sulfide. Approximately 120 hours after being introduced into low oxygen water the animals begin to turn black. The coloration begins at the mouth end and slowly proceeds aborally during a period of several days. Dissection of such animals reveals deposits of dark material on the mesenteries and the inside surface of the body wall, in addition to the noticeable odor of hydrogen sulfide. This color change is not accompanied by death and subsequent decomposition. Upon return of intact black *H. producta* to running seawater the coloration disappears in less than one day, and the animal resumes its normal activities.

During exposures to low oxygen conditions, *H. producta* undergoes marked volume changes. Several days after introduction into such conditions the animals become inflated and distended to a volume about 10 times their body volume in running seawater. There is a threefold increase in body length and a twofold increase in body diameter. Body volume increases occur in *M. senile* under similar conditions, but are less in magnitude. Volume increases in both species are reversed on return to running seawater.

Anatomy

Haloclava producta placed freely in running seawater or anesthesized in $MgSO_4$ is considerably shorter (3–5 cm long) and thicker (0.8–1.0 cm in diameter) than individuals in burrows. There are 20 cylindrical tentacles about 5 mm long and 0.5–1 mm in diameter. Although largely insensitive to touch, the tentacles are retracted under extreme conditions (*e.g.*, chronic exposure to anoxia).

TABLE I

Thermal history and number of animals in each experimental group for measurement of metabolic response

Acclimation temperature (°C)	Experimental temperature (°C)			
	10	17	20	22.5
10	1	4		6
17		8*		
20	3	1	2	5
22.5				2

* Metabolic response determined on same animals after 24 hr exposure to anoxia.

The column is marked by 20 faint lines indicating the insertions of 20 macrocnemal mesenteries. In sectioned material (Fig. 3A) the mesenteries are seen as very thin and delicate septa with small longitudinal retractor muscles. Between the externally visible mesentery insertions are 20 vertical rows of adhesive papillae extending from the oral margin to a variable point down the column, usually one third to one half the total body length. There are 400–600 of these papillae visible on preserved specimens, depending on the particular state of contraction of the specimen. In cross section (Fig. 3A) the papillae appear as hemispherical blisters on the sides of the animal where both the epidermis and the gastrodermis are thinner than elsewhere along the body wall. The body wall thickness of *H. producta* as determined from sections is 141 μ; the thickness at the apex of representative adhesive papillae is 63 μ.

Repeated efforts to detect ciliary currents associated with the tentacles and oral disc of *H. producta* were unsuccessful in both burrowed and non-burrowed animals. Ciliary currents associated with the actinopharynx and mesenterial filaments, however, were easily and repeatedly observed.

Detailed anatomical descriptions of *M. senile* are given elsewhere (Stephenson, 1935; Batham and Pantin, 1951; Hand, 1955b), so only observations pertinent here are discussed below. The general body shape of *M. senile* is considerably less vermiform than that of *H. producta*. The column is smooth with only occasional perforations in the form of cinclides. Tentacles are longer, thinner, and considerably more numerous than those of *H. producta*. They are, in addition, much more readily retracted in response to mechanical stimulation.

The internal organization of *M. senile* (Fig. 3B) is both more complex and more bulky than that of *H. producta*. There are at least 4 cycles of mesenterial septa (96 individual septa) in small individuals. This is five times as many as in *H. producta*. These individual septa are slightly thicker than those in *H. producta* and the cross-sectional area of each longitudinal retractor muscle in the primary and secondary cycles (the 24 largest retractors) is at least 5 times that of the *H. producta* retractors. These numerous mesenterial septa tend to compartmentalize the gastrovascular cavity of *M. senile* to a much greater extent than in *H. producta*. In addition, there is simply a greater bulk of tissue inside *M. senile*. Our sections of *M. senile* are of an individual with a column diameter of 5 mm, the same size as *H. producta*. Hand (1955b) indicates that larger *M. senile* may have several more mesenterial cycles, and since each additional cycle doubles the number of septa, the internal complexity of *M. senile* increases with size as a geometric progression. *H. producta*, on the other hand, has a fixed number of mesenteries (Verrill, 1899).

TABLE II

Allocation of surface area

Species	Diameter (mm)	Length (mm)	Number	Surface area (mm^2)	%Total surface area
Haloclava producta					
Body wall	6	100		1884	85.3
Tentacles	1	5	20	314	14.7
Metridium senile					
Body wall	30	40		3768	44.4
Tentacles	0.5	15	200	4710	55.6

The easily observable ciliary currents on the tentacles of *M. senile* are directed aborally from the tentacle bases. The oral disc is marked by currents from the mouth running peripherally. The actinopharynx and mesenterial filaments are ciliated and circulation of material along the inside surface of the tentacles was observed in favorable preparations. The body wall thickness is 200–235 μ, almost twice that of non-extended *H. producta*, and an order of magnitude thicker than burrowed *H. producta*.

Several major features of the anatomical organization of the two species are strikingly different. Even the most casual observations under conditions simulating the natural habitat show that anatomical allocation of surface area is distinctly different. Calculations of surface areas of body wall and tentacles for living specimens of each species under natural conditions are shown in Table II. In calculations for *H. producta*, body wall dimensions are given for the least degree of vermiformicity observed in burrowed animals. For *M. senile*, the body wall dimensions are those of a typical expanded animal and the estimated number of tentacles is intentionally conservative. Stephenson (1935) indicates that the number of tentacles in a crown of *M. senile* ranges up to 700. In both species areas are calculated for epidermal surfaces only. It is clear from Table II that the major contribution to total external surface area in *H. producta* comes from the body wall, and that the tentacle component is rather small. In direct contrast, tentacles of *M. senile* comprise a rather significant component of the total surface area, and in fact their contribution exceeds that of the body wall.

A second major difference in anatomical organization between the two species involves the complexity of their respective internal structure. *M. senile*, which has a greater number of larger mesenteries, is both more compartmentalized and has internalized a greater percentage of its total bulk.

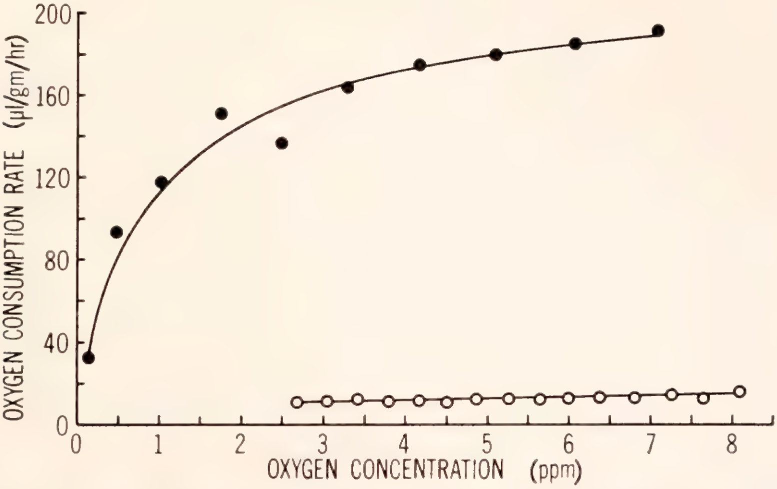


FIGURE 4. The relationship between oxygen consumption rate and oxygen concentration in two individuals of *Metridium senile*. Curves eye-fitted to raw oxygen consumption data.

Oxygen consumption rate and activity at air saturation

The frequency of spontaneous columnar contractions in *Haloclava producta* at 17° C is comparable to analogous measurements at the same temperature in both *Metridium senile* (Batham and Pantin, 1950a) and *Diadumene leucolena* (Sassaman and Mangum, 1970). The oxygen consumption rate of a 1 g *H. producta* at 17° C and air saturation is approximately 30 µl/g/ hr, considerably lower than the rates for *M. senile* (100 µl/g/hr) and *D. leucolena* (50 µl/g/hr) (Sassaman and Mangum, 1970).

Metabolic response to diminishing concentrations of oxygen

Gradual decreases in the oxygen content of ambient water at concentrations near air saturation are reflected in gradual decreases of oxygen consumption rate in *Metridium senile.* At lower concentrations, the decrease in oxygen consumption rate becomes more pronounced per unit decrease in oxygen concentration (Fig. 4). In this respect *M. senile* resembles the many other organisms which show such partial or imperfect metabolic regulation (C. P. Mangum, 1970; Mangum, Kushins and Sassaman, 1970; Kushins and Mangum, 1971). Oxygen consumption rate is better correlated ($P < 0.001$) with the logarithm of oxygen concentration than the linear axis (Table III). The slope of this semilogarithmic regression (b) is therefore an index of the degree to which oxygen consumption varies with oxygen concentration over the range of air saturation to zero. The coefficient is not a constant, but is highly variable between experiments, ranging from a high of 93.0 to a low of 6.4. When b is high, oxygen consumption rate is greatly affected by changes in oxygen concentration and the metabolic response curve shows partial conformity (Fig. 4, closed circles). When b is low, the oxygen consumption rate is little affected by changes in oxygen concentration (Fig. 4, open circles). The use of b as a quantitative index of sensitivity is valid only if its variation is not a consequence of time dependence in a closed monitoring system, or that oxygen consumption is not influenced by the rate of change in oxygen concentration. Figure 5 shows the relationship between b and k. If all 40 experiments are considered as a group, it is readily evident that there is no systematic relationship between oxygen sensitivity (b) and the oxygen depletion constant (k) ($P \geq 0.05$). At any fixed value of k there is no specified corresponding value of b. We regard the data in Figure 5 as strong evidence for the lack of a relationship between b and k and hence as strong support for the use of b as a quantitative measure of oxygen sensitivity. Thus, the observed variation in b is not an artifact, and the question arises as to whether this variation is random or is systematically correlated with environmental factors.

TABLE III

Fit of linear and semilogarithmic regression lines to relationship of oxygen consumption versus *oxygen concentration according to Student's* t

Species	N	Mean value (± S.E.) of coefficient of determination (r^2)		*P* level
		semilog	linear	
Metridium senile				
pre-anoxia	32	0.88 ± 0.11	0.75 ± 0.07	<0.001
post-anoxia	8	0.81 ± 0.15	0.69 ± 0.11	>0.10
Haloclava producta				
pre-anoxia	5	0.82 ± 0.19	0.80 ± 0.18	>0.80
post-anoxia	5	0.92 ± 0.21	0.93 ± 0.25	>0.80

In comparing the various b values from the various experimental groups (Table I) the following relationships were observed: (1) Among groups acclimated to a common temperature, b increases systematically with experimental temperature: (2) Among groups at a constant experimental temperature, b varies inversely with acclimation temperature; (3) Within individual experimental groups, b is negatively correlated with weight ($P < 0.01$); and (4) Between the two groups with identical thermal histories, high b values are obtained from those animals which were previously exposed to anoxia. In cases (1)–(3), high b values are correlated with conditions known to be associated with high oxygen consumption rates at or near air saturation (Sassaman and Mangum, 1970). In the fourth case, exposure to anoxia also brings about a subsequent elevation in oxygen consumption rate at high oxygen concentration (see below). These various correlations between high b values and elevated oxygen consumption rates at high oxygen concentrations are not coincidental. As shown in Figure 6, b is positively correlated with oxygen consumption rate at 8 ppm ($P < 0.05$). The oxygen consumption rates plotted in Figure 6 are values taken from semilogarithmic regression lines and the value of 8 ppm deviates less than 2.5% from air saturation for all the experimental groups.

Figure 6 clearly shows a linear relation between b (or absolute oxygen sensitivity) and oxygen consumption rate at air saturation (R) such that:

$$b = \frac{R}{1.6} \tag{1}$$

or:

$$\frac{(\Delta\ \mu\text{l/g/hr})}{(\Delta \log \text{ppm})} = \frac{(\mu\text{l/g/hr}) \text{ at air sat.}}{1.6} \tag{2}$$

which yields:

$$\frac{(\Delta\ \mu\text{l/g/hr})}{(\mu\text{l/g/hr}) \text{ at air sat.}} = \frac{(\Delta \log \text{ppm})}{1.6} \tag{3}$$

Thus, for any fixed reduction of oxygen concentration, Δ log ppm becomes a constant and:

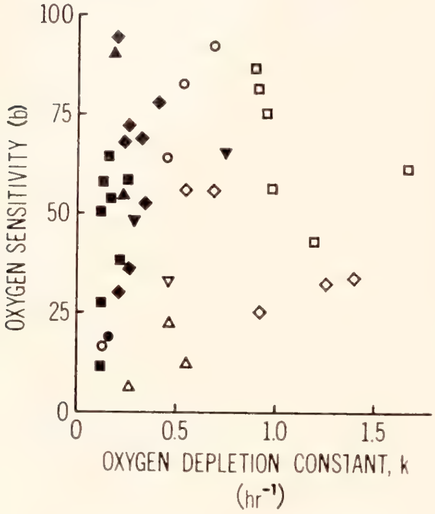
$$\frac{(\Delta\ \mu\text{l/g/hr})}{(\mu\text{l/g/hr}) \text{ at air sat.}} = (\text{constant})\ (0.63) \tag{4}$$

Application of equation (4) to the raw data indicates explicitly that, under the conditions employed, *M. senile* responds to a given change in oxygen concentration by changing its oxygen consumption rate by the same percentage which we call c. Thus, although different individuals have different absolute oxygen sensitivities (b) as indicated in Figure 4 and Figure 6, all the animals have the same relative oxygen sensitivity (c). A particular reduction in oxygen concentration elicits the same percentage reduction in oxygen consumption rate in all animals, regardless of thermal history, weight, or previous exposure to anoxia.

Metabolic response to prolonged anoxia

Decreases in the oxygen content of ambient water are reflected in decreases in the oxygen consumption rate of *Haloclava producta*. Perhaps due to the smaller number of observations, we cannot clearly distinguish the best fit (Table III). Semilogarithmic and linear relationships are not significantly different ($P > 0.80$). For comparative purposes, semilogarithmic regression coefficients are used here. Although insufficient data were collected to permit a detailed comparison of the relationship between absolute oxygen sensitivity (b) and oxygen consumption rate at air saturation (R) in *H. producta versus Metridium senile,* several interesting observations can be made.

In both species, measurable oxygen consumption ceases at oxygen concentrations above zero. Since cessation persists for such long periods, we believe that a switchover to anaerobic respiration occurs in both *M. senile* and *H. producta,* despite the recent contention (Beattie, 1971) that estuarine anemones do not shift to anaerobic pathways during anoxia. The oxygen concentrations at which oxygen consumption ceases are given in Table IV. The residual concentration in *M. senile* is considerably lower than that in *H. producta,* the difference being significant at $P < 0.001$. In *H. producta* residual concentration is not significantly correlated with body weight, whereas in *M. senile* smaller animals tend to have higher residual concentrations ($P < 0.01$). The difference between the two species may not be real, however, since the number of observations on *H. producta* is smaller. In both species there is a significant ($P < 0.01$) compensatory reduction in residual concentration following anoxia.



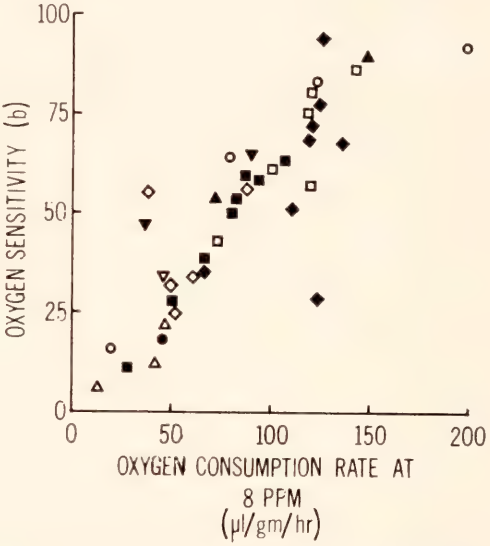


FIGURE 5. The relationship between absolute oxygen sensitivity and the rate constant for oxygen depletion in *Metridium senile.* Closed symbols for animals acclimated to and measured at the following temperatures: circles, 10° C; squares, 17° C; triangles, 20° C; inverted triangles, 22.5° C; diamonds, 17° C (after 24 hr anoxia). Open symbols: circles and squares represent animals acclimated to 10° C and acutely measured at 17° and 22.5° C, respectively; triangles, inverted triangles and diamonds represent animals acclimated to 20° C and subsequently measured acutely at 10°, 17° and 22.5° C, respectively.

FIGURE 6. The relationship between absolute oxygen sensitivity and oxygen consumption rate at 8 ppm in *Metridium senile.* Symbols as described for Figure 5.

Upon re-introduction into air saturated seawater (following a 24 hr period of anoxia), *M. senile* shows the biphasic metabolic response illustrated in Figure 7. The initial phase is marked by a rapid increase in oxygen consumption rate with time, the second phase being characterized by a gradual decrease in oxygen con-

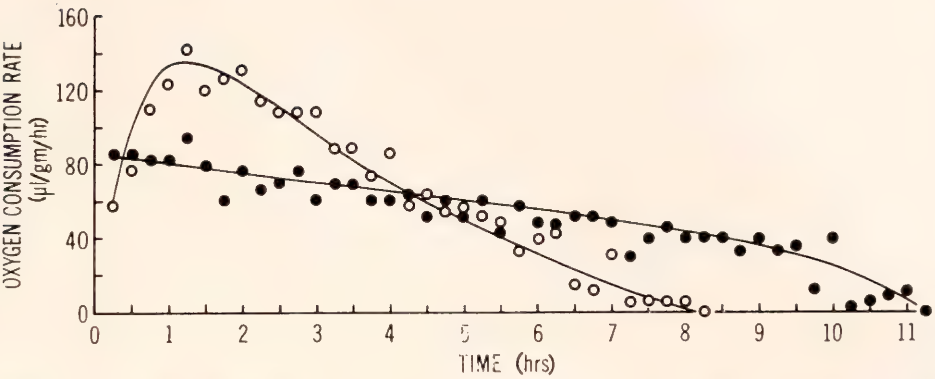


FIGURE 7. Oxygen consumption rate as a function of time in an individual *Metridium senile* before (closed circles) and after (open circles) 24 hr exposure to anoxia (curves eye fitted).

sumption rate as the ambient oxygen concentration decreases. Lag time (the time required to reach maximum oxygen consumption rate) varies in *M. senile* from several minutes to more than one hour and is correlated with animal weight ($P < 0.05$). In only one instance was a lag time recorded in *H. producta.* Since the lag was less than 5 min, it may have been caused by recorder stabilization, but in any event it was far shorter than any lag observed in *M. senile.*

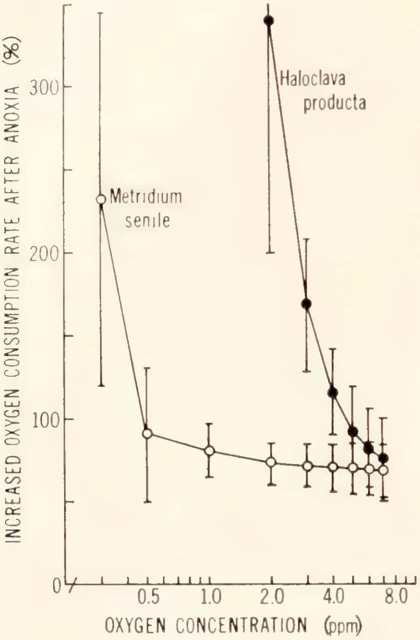
Oxygen consumption rates following anoxia significantly increase in both species. Figure 8 shows the per cent increase in oxygen consumption rate following anoxia as a function of the oxygen concentration. In both species the per cent increase is significantly different from zero at all oxygen levels above 1 ppm ($P < 0.05$) and it varies inversely with oxygen concentration. Both curves in

TABLE IV

Residual oxygen concentrations before and after exposure to low oxygen conditions

Species	Wet weight (g)	Residual concentration before exposure (ppm)	Residual concentration after exposure (ppm)	Change in residual concentration (ppm)
Metridium senile				
	1.86	0.79	0.52	−0.27
	1.94	0.21	0.00	−0.21
	1.95	0.78	0.00	−0.78
	2.68	0.34	0.17	−0.17
	3.26	0.30	0.18	−0.12
	3.38	0.24	0.16	−0.08
	4.09	0.79	0.04	−0.75
	5.00	0.17	0.00	−0.17
	5.66	0.25	0.00	−0.25
Haloclava producta				
	0.392	2.14	0.37	−1.77
	0.474	0.98	0.08	−0.90
	0.521	1.80	0.20	−1.60
	0.611	1.59	0.63	−0.96
	0.635	0.73	0.05	−0.68

Figure 8 asymptote at oxygen concentrations where oxygen consumption rates become very low (Table IV). It is only at these asymptotes that the percent increase in oxygen consumption becomes less than significant ($P > 0.05$). The absolute increase in oxygen consumption rate following anoxia is shown as a function of oxygen concentration for both species in Figure 9. The absolute increase is significantly different from zero at all oxygen concentrations in both species indicating that the lack of significance at 1 ppm in Figure 8 is a statistical artifact. Interestingly enough, the variation in absolute increase between individuals of the same species is surprisingly low.



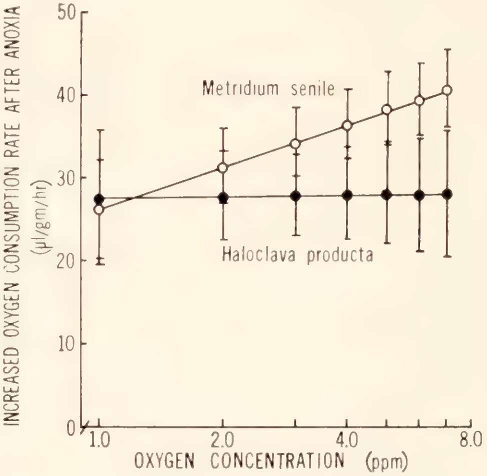


FIGURE 8. The relative increase in oxygen consumption rate after 24 hr anoxia as a function of oxygen concentration (vertical bars ± S.E.).

FIGURE 9. The absolute increase in oxygen consumption rate after 24 hr anoxia as a function of oxygen concentration (vertical bars ± S.E.).

The changes in absolute oxygen sensitivity (b) of both species after exposure to 24 hr anoxia are given in Table V. In *M. senile* there is a significant increase in b ($P < 0.05$) in 4 of 8 anemones. In 7 of the 8 individuals there is an increase in b, and in the one instance where a decrease is noted, the change is non-significant ($P > 0.05$). It seems that the general response of *M. senile* to prolonged exposure to anoxia includes a subsequent increase in absolute oxygen sensitivity (b), although the response is not entirely invariant. However, as we have already shown (Fig. 6 and Equation 4) the relative sensitivity to reduced oxygen concentration (c) does not change after exposure to anoxia.

In *H. producta*, on the other hand, the changes in oxygen sensitivity following anoxia are much more variable. Absolute oxygen sensitivity (b) increases significantly ($P < 0.05$) in one instance, decreases significantly ($P < 0.05$) in two instances, and remains relatively unchanged in the two remaining instances (Table V). This inconsistency of response in *H. producta* is very similar to that in *Nassarius obsoletus* and *N. trivittatus*, two snails which often burrow into low oxygen mud (Kushins and Mangum, 1971. The inconsistent response of *H. producta* contrasts markedly with the more stereotyped increase in absolute oxygen sensitivity in *M. senile*. Furthermore, since oxygen consumption rate consistently increases after anoxia in *H. producta* (Fig. 9) but absolute oxygen sensitivity (b) does not, it is clear that there is no single relative oxygen sensitivity (c) for *H. producta*.

TABLE V

Change in absolute oxygen sensitivity following anoxia

Species	Oxygen sensitivity before anoxia (b)	Oxygen sensitivity after anoxia (b)	Change (b)	Statistical significance (P)
Metridium senile				
	53.74	72.48	18.74	<0.05
	57.61	94.02	36.41	<0.05
	11.44	35.97	24.53	<0.05
	27.13	77.22	50.09	<0.05
	57.99	69.50	11.51	ns
	63.76	68.10	4.34	ns
	37.74	51.75	14.01	ns
	50.57	29.19	−21.38	ns
Haloclava producta				
	38.07	97.44	59.31	<0.05
	42.78	46.37	3.59	ns
	111.55	76.55	−35.00	<0.05
	38.55	22.78	−15.77	<0.05
	85.33	82.55	−2.78	ns

DISCUSSION

Metridium senile is continuously bathed in water of relatively high oxygen content (140–160 mm Hg) except during brief periods of intertidal exposure. Although Parker (1922) has shown measurable CO_2 production in *M. senile* under aerial conditions, the relationship between submerged and aerial aerobic metabolism in this species is not known. In any event, the prevailing oxygen content of the *M. senile* environment is constantly at or near air saturation.

It is clear from Figure 2 that irrigation by *H. producta* is sufficient to maintain quite high pO_2's in artificial burrows. In natural burrows it is doubtful that loss of burrow oxygen to surrounding low oxygen sediments is sufficient to lower appreciably the oxygen levels of the burrow while the animal is irrigating. The occupation and irrigation of a burrow, however, would tend to require certain behavioral and morphological adaptations which might be evident in a comparison between *M. senile* and *H. producta*. In addition, the oxygen content of the *H. producta* environment might well be less constant and less predictable than that of *M. senile*. Conditions which interrupt irrigation behavior lead quite rapidly to low burrow pO_2's. Although our kymographic recording suggest long periods of uninterrupted irrigation in a shaded area of the laboratory, irrigation may not be continuous in the field. Hargitt (1907) has described the importance of negative photo-tropisms in *H. producta* behavioral patterns. In addition, periods of intertidal exposure and storm shifted sediments would force cessation of burrow irrigation. Given these considerations we might expect to find two types of adaptations in *H. producta* relative to *M. senile:* (1) those facilitating extraction of oxygen from an irrigation current, and (2) those allowing for a rapid "rebound" from fortuitous periods of anoxia.

The major contribution to surface area in *H. producta* comes from the body wall whereas in *M. senile* the tentacles are of greater importance. The dichotomy becomes even more striking when we note that the body wall of *H. producta* in its burrow is at least one order of magnitude thinner than that of *M. senile*, that the body wall of *H. producta* is studded with several hundred even thinner walled papillae, and that *H. producta* shows no apparent tentacular ciliation whereas *M. senile* does. These morphological features suggest a major difference in the site of oxygen diffusion into the two species: exchange in *M. senile* is typically localized to the tentacles but it is spread out more linearly in the elongate *H. producta*.

Both *M. senile* and *H. producta* show volume increases on exposure to low oxygen conditions, but the response is much more stereotyped and extreme in *H. producta*. Such marked volume increases have two major effects on the respiratory properties of *H. producta*. First, the surface area to weight ratio is greatly increased, allowing a greater area for diffusion. Secondly, the diffusion distance across the body wall is reduced, thereby increasing body wall permeability. Both of these effects greatly increase the oxygen exchange capacity of *H. producta* upon resumption of burrow irrigation, as suggested by the rarity or absence of a lag time.

A second major anatomical difference between *M. senile* and *H. producta* involves the complexity of their respective internal organizations. As in most anthozoans, the enteron is open and continuous, and only partially compartmentalized by the mesenterial septa. As Stephenson (1921) has pointed out, ciliary activity of the mesenterial filaments probably helps maintain the continuity of the enteric fluid by keeping apart adjacent septa. Nevertheless, the degree to which the enteron is compartmentalized may be related to mesenterial organization. Internal mixing of the open enteron and access of enteric water to spaces between mesenteries may be facilitated by the lack of complex organization in forms such as *H. producta*.

The observation that contracted pennatulids, in which enteric fluid circulation is greatly decreased, have low metabolic rates (Brafield and Chapman, 1965) suggests that the major site of oxygen consumption is gastrodermal. In actinians the bulk of tissue is gastrodermal; specifically, half of the body wall and tentacles, and all of the mesenteries are gastrodermal (Stephenson, 1928), if mesoglea is discounted. We have no data from this or previous studies (Sassaman and Mangum, 1970) to suggest that appreciable amounts of oxygen are exchanged gastrodermally in actinians. In our continuous recordings of oxygen consumption there is no indication of any periodic rapid decrease in oxygen concentration as has been demonstrated in pennatulids (Brafield and Chapman, 1967) and renillids (Chapman, 1972). Furthermore, it is unlikely that continuous peristaltic irrigation waves, such as we record from *H. producta*, are generated by an animal continuously varying its body volume (and hence hydrostatic pressure) by ventilation of the gastrovascular cavity. In addition, the frequency of large scale emptying of the gastrovascular cavity of *M. senile* is low (Batham and Pantin, 1950b) and it is unlikely that the volume of water exchanged during minor columnar contraction carries an appreciable amount of the total requisite oxygen into the gastrovascular cavity for direct transfer to the gastrodermis.

Actinians seem to be faced with the problem of transporting and delivering oxygen, which is procured primarily at an epidermal site, to a primarily gastrodermal site of consumption. Morphological adaptations in a burrowing anemone might therefore tend to maximize the epidermis/gastrodermis ratio and maximize gastrovascular continuity. In addition, one might expect enhancement of gas exchange via thinning of the body wall and a shift in the site of oxygen exchange to the body wall as adaptations for rapid re-oxygenation of gastrovascular fluid in a burrow-irrigating anemone.

Vermiformicity and simplicity of internal organization are certainly not obligatory features of burrowing anemones. Indeed, there are structurally rather complex anemones which exhibit the burrowing habit (Hand, 1955a; D. C. Mangum, 1970), and in some other anthozoans direct ventilation of the gastrodermis seems a viable alternative (Parker, 1920; Brafield and Chapman, 1967). In fact, within the sea anemones there is a phylogenetic component associated with many of these morphological correlates of burrowing. Halcampids and haloclavids, both of which show vermiformicity and simplicity of internal organization, are more closely related to each other phylogenetically than either is to some of the more structurally complex burrowing anemones (Stephenson, 1921). Nevertheless, the kinds of structural features emphasized here cut across phylogenetic lines. Vermiformicity and internal simplicity are characteristic of edwardsids, an anthozoan line distinct from that of the halcampids and haloclavids (Stephenson, 1921). Parallel construction is found in the glycerid polychaetes in which typical annelid vermiformicity is accompanied by metameric distribution of the gills and loss of intersegmental setpa. A very striking parallelism occurs in such synaptid holothuroids as *Leptosynapta*, in which the perivisceral coelom is almost totally uncompartmentalized via loss of the bulk of the hemal and water vascular systems, the respiratory trees are lost, the surface area of the tentacles greatly reduced and the body elongated. Similar vermiformicity and simplicity of internal organization is characteristic of the meiofauna inhabiting the interstitia of marine sediments (Swedmark, 1964). The modifications which we see in *H. producta* are apparently representative of a general pattern or strategy of adaptation to infaunal existence and they are not a specific solution employed by one group of sea anemones.

Comparison of physiological properties of *H. producta* and *M. senile* support the same general conclusion which have emerged from other studies comparing species from stable high oxygen environments with closely related forms living in lower or less predictable oxygen environments. Although both species survive anoxia for periods considerably in excess of those generated by tidal cycles, survival of *H. producta* is at least twice that of *M. senile*. In addition, at comparable levels of columnar activity and oxygen saturation, *H. producta* has a lower oxygen consumption rate than *M. senile* of the same body size. There is no apparent correlation between environmental oxygen level and absolute oxygen sensitivity. The variation in absolute oxygen sensitivity (*b*) confirms the findings of numerous studies which have shown that there is no constant value in a species for the relationship between oxygen consumption rate and oxygen concentration (Helff, 1928; Hiestand, 1931; Hyman, 1932; Lindeman, 1935; Thomas, 1954; Van Dam, 1954; Wiens and Armitage, 1961; Eriksen, 1963; Nimura and Inoue, 1969; Thompson and Pritchard, 1969; Bayne, 1971). Of particular interest, however, is the finding of a single relative oxygen sensitivity (*c*) in *M. senile* which is independent of acclimation temperature, experimental temperature, weight and previous exposure to anoxia. A comparable constant is not found in *H. producta*. Its absence may characterize species inhabiting low or unpredictable oxygen environments, but interpretation of the ecological correlation is not yet obvious.

This study was supported by research grant GB-20335 from the National Science Foundation. We thank R. J. Hoffmann, G. B. Johnson, L. R. Moore and W. Van Winkle for critically reading the manuscript.

SUMMARY

1. Aerobic metabolism in *Haloclava producta*, a burrowing sea anemone, is largely or wholly dependent upon the oxygen supply in the water overlying its burrow.

2. This superficial water is brought through the burrow by an irrigation cycle of peristaltic waves. About 33% of the oxygen in the irrigation current is withdrawn by the animal.

3. Externally *H. producta* is exceedingly vermiform, studded with numerous thin walled hollow blisters and body surface area largely consists of the columnar body wall. *M. senile*, in comparison, is more robust, relatively smooth walled, and most of its total surface area is tentacular. Internally, the body wall of *H. producta* is an order of magnitude thinner than that of *M. senile*, and *H. producta* has fewer and smaller mesenterial septa and longitudinal retractors.

4. These morphological considerations suggest that vermiformicity and simplicity of internal organization are adaptive to low or unstable oxygen environments. The modifications associated with *H. producta* are not uniquely actinian, and are paralleled in completely unrelated phyla; thus they may represent a general mode or pattern of adaptation to burrow existence.

5. The morphological adaptations seem to be correlated with efficient use of the oxygen in the irrigation current, and with rapid restoration of internal pO_2's following transient periods of anoxia.

6. *H. producta* is much more resistant to prolonged anoxia than *M. senile*, and it has a lower oxygen consumption rate at air saturation.

7. After exposure to anoxia, both species show a compensatory increase in the fraction of dissolved oxygen which they can remove from a closed system.

8. After exposure to anoxia, both species show an increased rate of oxygen consumption (both in relative and absolute terms) over wide ranges of oxygen concentration.

9. Different individuals of *M. senile* have very different absolute metabolic response curves to diminishing oxygen concentration, but all individuals examined showed the same relative decrease in oxygen consumption rate over a given change in oxygen concentration. This relative change is independent of acclimation temperature, experimental temperature, weight and previous exposure to anoxia. Different individuals of *H. producta* also have different absolute metabolic response curves to diminishing oxygen concentration, but a single relative response comparable to that in *M. senile* does not exist.

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THE EFFECT OF COPPER (II) ON SURVIVAL, RESPIRATION, AND HEART RATE IN THE COMMON BLUE MUSSEL, *MYTILUS EDULIS*

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Although copper in trace quantities is required by living organisms, small increments above the required level are highly toxic. Copper looms most importantly in the Crustacea and Mollusca where it forms a part of hemocyanin, an oxygen transport pigment, about which much remains unresolved (Wilbur and Yonge, 1965). Hemocyanin has been identified in all classes of the Mollusca except the Bivalvia which does not appear to have a functional oxygen-carrying pigment (Morton, 1958). Copper is an important constituent of both tyrosinase and cytochrome oxidases and as such presumably has a universal distribution in all classes. Prytherch (1931) showed that copper is required for the attachment and metamorphosis of the oyster, *Crassostrea virginica.* The physiological changes accompanying toxic Cu(II) levels have not been deeply examined. These effects are of current relevance in northeastern New England where a heavily mineralized coastal belt is being returned to active copper mining with discharge of mineral rich washings into coastal waters. In 1968 mining began at Cape Rosier (46° 21′ N, 68° 47′ W) on Penobscot Bay, Maine and new operations are planned for 1972 in nearby Blue Hill Bay.

Materials and Methods

Collection and maintenance of animals

Animals for respiratory work, heart rate studies, and copper concentration studies were collected at Lamoine, Maine, between May 1970 and January 1971, from water ranging in temperature from − 1 to 15° C. Animals were returned to the laboratory and kept in 10 gallon tanks, continuously aerated, which were maintained at 10° C. Carboys of water were also taken since the use of artificial sea water (Instant Ocean, Aquarium Systems, Inc.) was found to be unsatisfactory. Those animals in the synthetic mixture consistently showed a higher degree of valve closure than those in natural sea water. Redistilled water was added to maintain the specific gravity at 1.025, and the pH was checked frequently. It varied from 8.0 to 8.2.

Copper measurements

The method chosen for extraction of copper was that of Riley and Sinhaseni (1958). The reagent for extraction and spectrophotometric determination of copper in sea water was 2;2′-diquinolyl in *n*-hexanol. The color of the cuprous diquinolyl complex was stabilized by addition of hydroquinone. 2;2′-diquinolyl reacts with copper in the cuprous state and in order to ensure that all copper is in this state, hydroxylamine hydrochloride is added. Absorbances were determined in a Bausch and Lomb Spectronic 20 spectrophotometer using a 1 cm light-path cell. Measurements were made at 540 nm and compared with a standard curve. The procedure was periodically checked by adding known amounts of Cu(II) to standard and experimental samples and determining recovery of the added Cu(II). Riley and Sinhaseni report a coefficient of variation of this method of 2.5% with sea water containing 0.027 mg/l copper. As employed one measures total copper.

Survival studies

Laboratory studies were carried out in an effort to determine the toxicity threshold for *M. edulis.* Duplicate glass tanks were set up for each of four copper concentrations (0.5, 0.3, 0.2 and 0.1 mg/l). Each tank contained 10 liters of sea water and 100 animals 1 to 2 cm in length. All tanks were well aerated, kept at 10° C, and the animals were not fed. It should be noted that this was a static system, and concentrations, as indicated were initial concentrations only. No attempt was made to maintain the levels of copper after the animals were introduced to the tanks. Ideally one would use a continuous flow system of fresh unfiltered sea water with a constantly maintained concentration of the metal as was done by Pringle *et al.* (1968). On the other hand, Raymont and Shields (1962) have concluded that although a constant flow is desirable in "long term" studies, there is no significant difference in the toxicity threshold (for *Mya*) as measured in the constant flow and static systems.

The copper salt chosen for stock solution was copper(II) chloride dihydrate. Working with *Nereis virens,* Raymont and Shields (1962) point out that while there may be slight differences in the toxicities of various cupric salts, the threshold toxicity is approximately the same.

Warburg respirometry

Respiration of *M. edulis* has been shown to vary with body size and temperature (Read, 1962), salinity (Schlieper, 1929; Maloeuf, 1938), seasonal change (Krüger, 1960), and reproductive state (Bruce, 1926). Reproductive state of the animals was not established in this study but animals were paired randomly from collections made on the same date.

The ratio of tissue wet weight to shell length was measured and found to be remarkably constant and essentially linear from 4–8 cm where the ratio was 1.2 g per cm. None of the animals were fed, and a starvation curve (holding time *vs.* endogenous respiration) was established. Animals were not used in respiration studies after a holding period of five days, although they did not show significant respiratory declines until the 10th day in the holding tanks. All measurements were at 10° C in a refrigerated Warburg respirometer and animals were selected to fit this equipment. Thirty animals, all 1.3 cm ± 0.1 cm were dried at 100° C for 18 hours. The dry weights (minus shell) had a range of 0.0117 g to 0.0129 g with a mean of 0.0121 g. No attempt was made to eliminate bacterial contamination since most anti-bacterial steps would involve the possibility of some toxicity effects on the mussels. Were the contents of each flask to be a successful bacterial culture then the bacterial contribution to total oxygen consumption would be an exponential effect. Beginning, in terms of biomass, several orders of magnitude below the molluscan biomass the bacterial oxygen contribution should not be perceptible over the time course of these experiments and should it intrude it should intrude exponentially. Control runs remain linear over the course of these experiments and tank water controls show no significant respiration vis-a-vis distilled water thermobarometers. Others have faced this same problem (Rottauwe, 1958; Read, 1962). The respiratory changes parallel the changes in other parameters measured and we are satisfied that the respiration measured does not include a significant bacterial contribution. The current investigation is based on a reading every 10 minutes and a mean based on one hour. Standard Warburg flasks (approximately 20 ml) contained four animals (1.3 cm each) in a volume of 4.05 ml of sea water.

Oxygen consumption may be looked upon as a convenient measure of energy transformation. The change in *M. edulis* from aerobic respiration may take place not only when poor conditions prevail but also, without apparent reason, in a well aerated environment (Rottauwe, 1958). It is important to use only those animals which appear to be in the same condition in the holding tanks. Only those with opened valves and extended mantles were chosen for this study. Observations on the condition of the animals were kept during all Warburg runs, and when any of the control animals closed down their valves, the readings from that flask were excluded from computation of the mean endogenous respiratory rate. Shaking stimulates shell closing.

None of the Warburg flasks were shaken to promote oxygen exchange across the air-water interface. According to Rottauwe (1958) above 50% air saturation at 19° C the rate of oxygen uptake of *M. edulis* becomes independent of the partial pressure. Thus without determining per cent air saturation of the sea water in the Warburg flasks, the assumption is being made that, during the two hour runs, the water is more than 50% saturated. This seems probable since all runs in this work are at 10° C, and at this temperature more oxygen is dissolved in the water than at 19° C. Furthermore, the greatest uptake recorded for a single flask is 30 μl/hr, and the filtering carried on by the animals inevitably promotes some gaseous exchange. The uptake of control animals remained linear over a period of several hours, even though at the maximum rate of oxygen consumption, 100% of the oxygen originally in the sea water (30.0 μl at air saturation) is used during the first hour of the experiment. On rare occasions, during preliminary work, the molluscs remained open while being shaken. The values obtained during these runs were the same as those found in the unshaken runs used in data collection.

Copper uptake studies

Attempts were made to measure copper uptake in the mussels and to determine the time course of the uptake. A tank system was set up with the same ratio of sea water to animal tissue as there was in the Warburg flasks. Each tank contained two liters of sea water (0.3 mg/l Cu(II)) and 44 animals, each 5.3 cm in length. Four animals were removed at 12, 22, 36, 48 and 72 hours and each mussel was then analyzed for copper. Control animals from sea water tanks containing no copper were also analyzed.

Detoxification mechanisms

Warburg runs were made as indicated with Cu(II) levels at 0.3 and 0.5 mg/ml. After 90 minutes the original organisms were removed and replaced with two new mussels, the flasks closed off, and oxygen measurement resumed. The procedure was repeated four times at each of the two Cu(II) levels. Depression of the respiratory rate would then be a measure of the toxicity remaining in the sea water.

A second procedure was also employed. Whole *M. edulis* were homogenized in sea water for five minutes using a standard teflon pestle tissue grinder in a glass vessel. They were then made up to 10% wt/vol with additional sea water. Of this homogenate, 0.2 ml was then added to 0.3 and 0.5 mg/l copper-sea water solutions whose volume was equivalent to the volumes used in the Warburg runs. The mixture was then incubated at 10° C in air for 90 minutes, centrifuged in a clinical centrifuge at 1800 g for 15 minutes, and the supernatant used as the incubation medium for the standard Warburg procedures already described. Each supernatant was tested for toxicity four times by this standard Warburg procedure. Depression of respiration would indicate the persistence of toxicity.

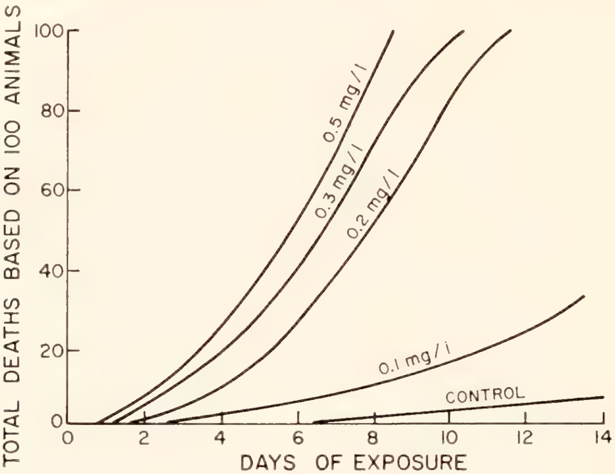

FIGURE 1. Toxicity experiment, 10° C.

Heart rate studies

Heart rates were obtained by cutting small (2 cm × 2 cm) windows in the left valve directly over the beating heart. All rates were measured at 10° C and only ventricular beats were counted. Schlieper (1955) has shown that when the shell of *M. edulis* closed, the heart rate may be reduced fourteenfold. The decreased heart rate as noted by Schlieper was not observed although there were frequent periods during which the heart completely ceased beating. In the present study several control animals were held in the closed position by placing rubber bands around the shells. No difference was noted in the heart rates of these animals or in the frequency or duration of non-beating periods. These periods did not appear to be related to valve closure and only periods of beating were included in calculation of heart rates during copper treatments.

RESULTS

Where two Cu(II) treatments are compared the *t* test at 5% level is employed while, with more complex comparisons, analysis of variance is employed and, where F is significant, Duncan's means discrimination test at the 5% level is the test of significance.

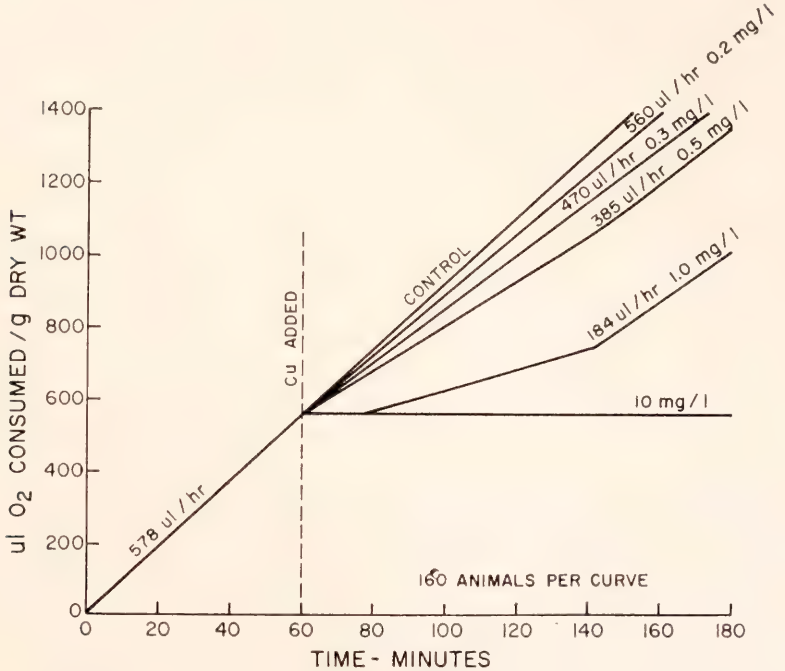

FIGURE 2. The effect of Cu(II) on *M. edulis* respiration at 10° C.

From Figure 1 it can be seen that the threshold toxicity appears between 0.1–0.2 mg/l. After seven days, 55% of the animals kept at 0.2 mg/l (initial concentration) were dead, whereas only 5% died after an equal period of time in 0.1 mg/l. Controls showed 1% mortality over the same time period. Comparable results were obtained by Marks (1962) working with *M. edulis*, Pringle *et al.* (1968) working with *Mya arenaria*, and Raymont and Shields (1962) working with *Nereis virens*. These authors set the toxicity threshold of copper for their respective animals at about 0.2 mg/l.

Results of respiratory studies are expressed as the mean of twenty runs (80 animals) at each concentration (Fig. 2). In all cases copper was added from the side arm of the flask at 60 minutes and the final volume of sea water in the flask was 4.05 ml with the indicated concentration of copper. In the case of controls, sea water was added from the side arm at 60 minutes. Studies were made with final copper concentrations of 10.0, 1.0, 0.5 and 0.2 mg/l.

Figure 2 shows the effects of copper on the respiration of *M. edulis*. Analysis of variance on the change in respiration (between $t = 80$ min and $t = 140$ min) shows significance at the 1% level. A Duncan's comparison of the mean changes in respiration for each of the plots shows no significance between the control and the 0.2 mg/l respiratory values. Depressions at the higher concentrations of copper are all significant.

At 10 mg/l the valves of the animals closed immediately after copper addition and remained closed for at least 15 hours, at which time the study was discontinued. At 1.0 mg/l the animals shutdown for approximately 15 minutes, after which the valves opened and the rate of oxygen consumption became 184 µl/hr. Approximately 90 minutes after addition of the copper there was a sudden increase in respiration until the rate approached the rate of oxygen consumption for the controls (578 µl/hr/g). At 0.5 mg/l there was no initial shutdown, but a recovery similar to that just described was apparent. At 0.3 mg/l there was neither a shutdown nor apparent recovery, and finally, at 0.2 mg/l copper, the change in respiration as measured over 2 hours at 10° C is not significantly different from control values. This data shows a dose response for the effect of copper on *M. edulis* respiration. Assuming a linear relationship, the equation for the change in respiration at 10° C over one hour as a function of copper concentration is:

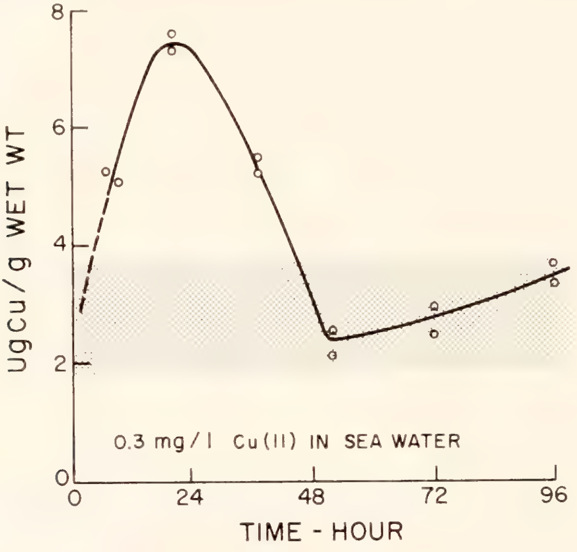

FIGURE 3. Cu(II) accumulation in *M. edulis* at 10° C.

$$\mu l\ O_2\ \text{consumed/g (dry wt)/hr} = 578\text{–}394\ (\text{Cu II conc. in mg/l}).$$

The results of copper uptake studies are indicated in Figure 3. Copper concentrations peaked at 22 hours exposure and then declined to the normal range.

The results of both methods employed in detoxification studies were similar. Animal respiration was within normal limits after treating sea water-copper solutions as previously indicated with either live animals or heat killed homogenate.

The heart rates of the points on the graph (Fig. 4) are the mean heart rates of eight animals at each concentration. Two standard deviations are indicated about each point. Copper was added only after regular beating was established. Controls showed regular beating for 24 hours. No change in heart rate at 0.2 mg/l copper was noted, but at higher concentrations a general dose response is clearly apparent within one hour.

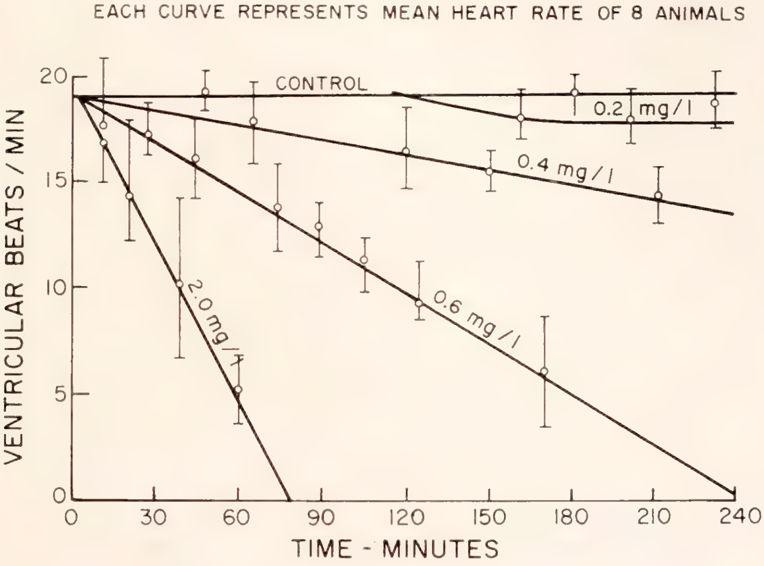

FIGURE 4. Effect of Cu(II) on *M. edulis* heart rate at 10° C.

DISCUSSION

In the sea, normal concentrations of copper lie between 0.01 mg/l in coastal waters (Atkins, 1932), and 0.003 mg/l in the open sea (Goldberg, 1963). Little is known of the copper cycle, and the distribution of this metal in the open sea, although Riley (1937) suggests that in deeper oceanic waters concentrations are significantly higher than at the surface. The Federal Water Quality Administration Report (1970) sampled 30 stations in Penobscot and Blue Hill Bays during 1967 and 1968 and reported water values ranging from 0.04 mg/l–0.37 mg/l around Cape Rosier area with the Blue Hill area twelve miles to the northeast ranging from 0.02–0.04 mg/l. Our own values, 12 determinations in each case, obtained in October 1970 give a mean of 0.0191 mg/l total copper in the water near the mine site and 0.0212 for our control area which was at Lamoine, Maine (44° 27′ N, 68° 17′ W), 25 miles to the northeast and marked by the same mineralization but lacking a mining history. Our values show no significant current difference between the two sites. *Mytilus edulis* was collected from the two sites and those from the Cape Rosier area contained 6.567 mg/kg (wet weight) copper, and the Lamoine controls 2.987 mg/kg. These differences are significant and consistent. Populations at Cape Rosier are sparse but apparently healthy and the control area shows only a higher population density. While the water levels of copper may fluctuate it is apparent that molluscs show the cumulative effects.

The literature is replete with studies showing that molluscs, polychaetes, and plankton have the ability to concentrate copper and other metals several thousandfold above ambient water levels. (Rose and Bodansky, 1920; Severy, 1923; Vinadograv, 1953; Chipman, Rice and Price, 1958; McFarren, Campbell and Engel, 1961; Galtsoff, 1964; Brooks and Rumsby, 1965; Pringle, Hissong, Katz and Mulawka, 1968). These papers indicate that all bivalves tested are proficient copper concentrators with *Crassostrea virginica* which reaches 19 mg/kg (Kopfler, 1966) being the most efficient and showing higher metal concentrations as one proceeds northward.

It is difficult to reconcile this data with exposure data in toxicology studies where *M. edulis* was killed in 12 hours by 0.55 mg/l copper (Ingols, 1955) and survived only 3–4 days following immersion of from 0.5–3.0 days in 0.14 mg/l copper (Clarke, 1947). The length of time that *M. edulis* could survive depended to some extent on its ability to keep its shell closed. In our toxicity studies 0.3 mg/l Cu(II) killed 55% of *M. edulis*, following an exposure of seven days. In Warburg studies, Cu(II) caused respiratory depression which became less obvious as the Cu(II) level declined. At the lower copper levels where this depression was clearly significant there was complete respiratory recovery despite continuous exposure. The live organisms had neutralized the copper effect. The alteration that occurred with time was not an adaptation of the organism but a change in the solution in some way. This is shown by the fact that new live organisms, added to the originally toxic solution after previously exposed organisms showed recovery, did not go through an adaptation stage. The solution in which they were placed was simply nontoxic. Using a heat-killed *M. edulis* homogenate one can also produce this detoxification of a Cu(II) solution.

All of these preceding data could be explained if the copper inside an organism were in some form that is nontoxic. One of the simplest ways for the Cu(II) to be effectively removed from the solution would be its passive binding with available organic ligands within the organism. One is inclined to this solution of the problem by the fact that a homogenized heat-killed extract can duplicate the action of the live organism.

During uptake studies at 0.3 mg/l Cu(II), the immediate response of mussels was to secrete copious amounts of mucous and this appeared consistently. Attempts to analyze this mucous, and the sea water into which it was released, failed due to formation of a stable emulsion during copper extraction procedures. However, Koringa (1952) found that cations can be absorbed on the mucous of the gills of *Crassostrea virginica*. Increased mucous secretion and subsequent binding may be another method of detoxifying sea water-Cu(II) solutions.

Clearing of the initial influx of Cu(II) is apparent from the uptake study (Fig. 3). It may be that the return to control copper levels is by clearing the initial internal copper (II) by transfer *via* excreted ligands, for example, by increased mucous secretion from the gills of the animal. When animals are placed in 0.3 mg/l copper, increased mucous production is observed. A second possibility is that copper is transferred to metabolic wastes and removed *via* the feces. Since live animals collected at Cape Rosier showed tissue levels (6.5 mg/kg) nearly as high as those attained in this experiment (7.5 mg/kg) one would not necessarily expect such levels to be lethal to *M. edulis*. Although animals taken in the Cape Rosier Region appear to be able to maintain 6.5 mg/kg in their tissues and remain in a viable condition, the animals in this experiment have been subjected to ionic copper.

This may indicate differences in the state of the copper in the two situations. From our data this is probably due to organic binding in the environment. Furthermore, since the toxicity studies show that death is caused in 100% of the animals exposed to 0.3 mg/l Cu(II), it is concluded that the lethal damage is done during the first 36 hours and is essentially irreversible.

When free Cu(II) is added to an ecosystem it must be presumed that organic binding occurs and that ingestion of such organically bound Cu(II) does not provide toxic levels of free Cu(II). Some organisms will be damaged by the free Cu(II) as it is bound since the initial binding presumably is the toxic step but, subsequently, members of the food chain may accumulate extraordinary levels of the organically bound copper without apparent effect. Total copper in a marine ecosystem is probably not the pertinent parameter of toxicity.

SUMMARY

1. The effects of Cu(II) on survival heart rate, and respiration in *M. edulis* have been determined.

2. Free Cu(II) is toxic to *M. edulis* causing respiratory and cardio-vascular depression and a toxicity threshold of 0.2 mg/l has been demonstrated.

3. Live animals can detoxify solutions of limited Cu(II) content.

4. Heat-killed homogenates are also effective in detoxifying solutions of limited Cu(II) content.

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AXIAL FILAMENT OF SILICIOUS SPONGE SPICULES, ITS ORGANIC COMPONENTS AND SYNTHESIS

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The rigid skeletal structures of the marine sponges in the class Demospongiae are silicious spicules bound together by protein fibers of collagen and spongin (Hyman, 1940). The regulation of growth and form in these animals involves controlling the synthesis and placement of these spicules. There is evidence that the growth pattern, while characteristic for a taxonomic group, is yet very sensitive to environmental factors related to current directions, velocity, and turbulence (Bidder, 1923). The production of spicules *de novo* is an intracellular secretory process which has received relatively little attention (Schröder, 1936; Jørgensen, 1944, 1947). We approach the problem of sponge spicule formation as a specific cellular activity which forms the basis of multicellular morphogenetic processes. To begin the analysis of spicule growth, the paper provides a procedure for monitoring silicious deposition by means of determinations of protein metabolism. I shall present evidence supporting two conclusions. (1) The axial filament in sponge spicules has a substantial protein component. (2) Synthesis of that protein occurs during spicule growth, and may be monitored by a radioisotope label in a protein precursor which becomes incorporated into the axial filament.

The desirable isotope, ^{32}Si, is not commercially available for radio-tracer studies of silicon metabolism. If (a) spicules could be freed of external organic matter, and if (b) synthesis of any intraspicule organic matter were correlated with silicious deposition, and if (c) that organic matter were largely protein, then (a) any organic matter in the HF-residue would be intrinsic intra-spicule material, and (b) any measure of synthesis of that material would be correlated with silicious deposition, and (c) protein precursors would label it. Thus, accumulation in spicule residue of label from a protein precursor could serve as an index of silicic acid deposition during spicule growth.

In the present work harsh cleaning procedures that would remove both organic and mineral contaminants from spicules prior to HF digestion, were coupled with two kinds of experiments that are reported here; (1) determination of the organic component of axial filament, (2) demonstration of incorporation of an appropriate precursor radioisotope into the axial filament in intact and cell suspensions of sponge.

MATERIALS AND METHODS

Preparation of spicules

The spicule preparation technique is based on several early observations: (1) that after digestion with HF, a microscopically visible filament remains (Bütschli, 1901); (2) that boiling concentrated nitric acid digests and dissolves protein and other organic matter from glass; (3) that the density of spicules (1.93) is intermediate between that of organic matter (*e.g.,* DNA, 1.7) and the more common mineral contaminants (*e.g.,* quarts, 2.4) (Vosmaer and Wijsman, 1905). The procedures leading up to carbon determination were designed to have no contact with carbon containing reagents (not even acetone or alcohol) to preclude any contamination with carbon residue. (The only exception is the use of the teflon filter after carbon had been detected on gold grid preparations.) For the analysis of axial filament composition, axial filaments were prepared from chunks of sponge (*Acarnus erithacus*) from the Pacific Coast near Los Angeles (Pacific Bio-Marine, Venice, California). This species was chosen because of its relatively high content of spicules and presence of a unique "palm tree" (cladostrongyle) spicule.

Pieces of *A. erithacus* were repeatedly heated for an hour to 85–90° in changes of concentrated nitric acid until the spicules were brilliant white and the acid did not discolor (usually 4 changes). The pellet of spicules was water washed. These spicules did not darken sulfuricdichromate (standard glass cleaning solution). Density gradients were prepared in 50 ml glass centrifuge tubes using $ZnCl_2$. A linear gradient was produced by mixing two stocks, one at density 2.1, the other at density 1.8, based on standard density tables for $ZnCl_2$ (Weast, 1961). A pilot run showed that the spicules banded at the position expected for density 1.93. Preparative density gradients of 35 ml were loaded with 5 ml of spicule slurry, *i.e.,* water wet spicules taken up in density 1.7 $ZnCl_2$. The gradients were centrifuged 30 minutes at 2000 rpm in a model PR2 centrifuge. The spicules were removed with the density 1.9 to 2.0 region, and repeatedly washed by centrifugation in hot water.

Preparation of spicule residues

Cleaned spicules were etched in three different ways for micro-analysis. (1) Spicules were partially etched as previously described (Schwab and Shore, 1971) on a paladium chip. (2) Spicules were loaded on a 400 mesh gold grid 3 mm diameter and etched completely in HF. The fluid was removed by evaporation providing a "whole non-volatile residue," or by suction (supported on a membrane filter) providing an "insoluble residue." (3) Gram quantities were etched in 12 N HF in teflon weighboats, filtered with suction on weighed teflon filters, and washed with suction on weighed teflon filters, and washed with suction in 1 N HF on the same filters, providing a "washed, HF-insoluble residue." The wash water was collected for analyses which are included in a separate report.

Preparation of samples for electron microprobe

Partly etched spicules on a coded palladium chip were coated with paladium by rotary vacuum evaporation. Spicule residues on 400 mesh gold grids were similarly coated with paladium or with aluminum. The grids were held on a coded copper chip by spring clips. The chips of copper and of paladium were placed in standard specimen holders and examined in an A. R. electron microprobe. The work reported here is based on operation of the probe in 4 modes: (1) secondary electron scattering image, (2) specific wave length x-ray image, (3) specific wave length x-ray intensity scan and (4) specific wave length x-ray point-count using a diamond standard for carbon. Qualitative inorganic analysis by means of x-ray spectral scan and specific wave length point counts are to be reported separately.

Preparation of sponge for isotope incorporation

Fingerlets of *Hymeniacidon sinapium* were aquarium-grown from sponge chunks collected in southern California (Pacific BioMarine, Venice, California) as previously described (Shore, 1971). Two modified synthetic sea-water media were used, CMF/Si and IOSW/Si. The former is based on a calcium and magnesium free balanced saline (Humphreys, 1963). The latter is based on a proprietary mixture (Instant Ocean Sea Salts made to density 1.023, Aquarium Systems, Wyckliff, Ohio). Both were made 0.5 mM with respect to silicate by the addition of $NaSiO_3$.

Cell suspensions were prepared from chunks of *H. sinapium* by mechanical disruption, and fractionated by isopycnic centrifugation in isosmotic sucrose. Sponge pieces, freed of large contaminants were sliced to pieces about 1–5 mm^3 and squeezed through nylon bolting colth in CMF/Si, 15 g damp sponge to 30 ml medium providing about 36 ml of suspension, the "whole" cell preparation. Portions of 5 ml suspension were layered over 30 ml sucrose gradients. These were linear between CMF/Si (density 1.023) and sucrose in distilled water (density 1.092). The concentration of surcrose was calculated to be isosmotic with sea water (density 1.023) from standard tables (Wolf and Brown, 1970). After 15 minutes centrifugation at 900 rpm in PR2 three fractions were collected. These are referred to as light cells, intermediate cells, and dense cells. They are, respectively, the top 10 ml, next 10 ml, and pellet. Each of these fractions was diluted in IOSW/Si before being suspended in IOSW/Si for incubation.

Isotope incorporation

Fingerlets or cell suspension were incubated in 35 mm petri dishes (Falcon Plastics) in a water bath at 18° C. Each dish contained 3 ml IOSW/Si. Radioactivity was added as a change of medium to which had been added 3H-1-leucine, specific activity 40 Ci/mM (Schwartz Bio-research, Orange, New Jersey) at a final activity of 4 μCi/ml. All fingerlets or cell suspensions in an experiment began the incubation at 18° at the same time. The incubation in isotope occurred for periods ranging from 15 minutes to 64 hours within the incubation at 18°. The schedule routinely provided for samples which had a short isotope incubation period during the first portion and others the last portion, of the longer incubation period. For example, there was a first-hour sample and a last-hour sample during the 64-hour incubation period. Incorporation was stopped by freezing on dry ice. A first extract, containing nucleic acids and amino acids, was obtained with 15 volumes of phenol-cresol (Kirby, 1968). Cleaned, non-radioactive spicules were added to cell suspension preparations at this point in extraction. After acetone washing, the phenol-cresol residue was digested in hot nitric acid to provide a second extract. The nitric acid residue was washed in water and digested in 1 N HF providing a third residue. Portions of the medium, the first and second extracts and the third residue were then counted in dioxane-fluors (Wannemacher, Banks and Wunner, 1965), in a liquid scintillation spectrometer (Nuclear Chicago, Mark II), to an accuracy of better than 5% at the 95% confidence level.

Amino acid analysis

A portion of a preparation of washed, HF-insoluble residue was dissolved in alkali and analyzed for protein according to Lowry, Rosebrough, Farr and Randall (1951). Portions of the same material were then taken up in water and dialyzed against water. One of these was subjected to oxidation with performic acid for 1 hour at 0° C and prior to hydrolysis. The others were directly hydrolyzed in 6 N HCl for 24 hours at 119 ± 1° C. The hydrolysates were each separated by ion exchange chromatography on a Beckman Amino Acid Analyzer. The ninhydrin-positive peaks were identified and the molar quantity of ach amino acid was calculated for the half-width by comparison with standards.

RESULTS

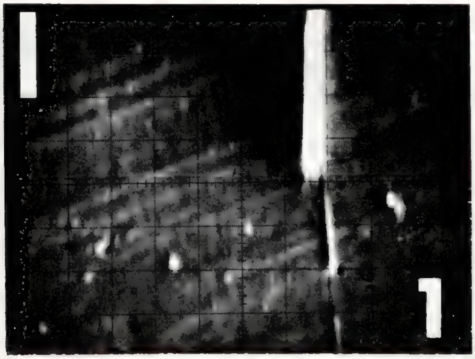
The results are presented in two sections, first the direct studies of organic composition, and second, the incorporation studies.

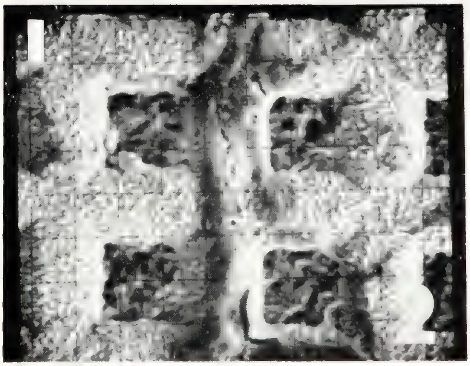
Organic composition of axial filament

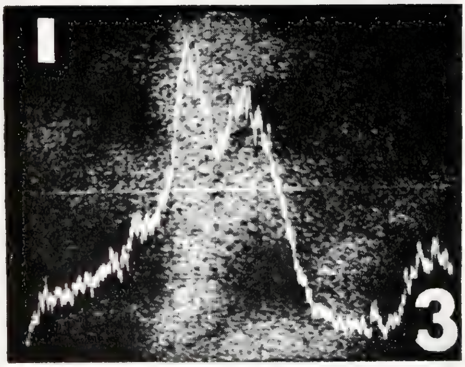
Single spicules, partially etched after the fashion figured in Schwab and Shore (1971) were subjected to qualitative chemical analysis by electron microprobe. Although the axial filament could be resolved in the secondary electron scattering image, Figure 1, the current required for chemical analysis destroyed these single axial filaments before carbon analysis could be reliably completed.

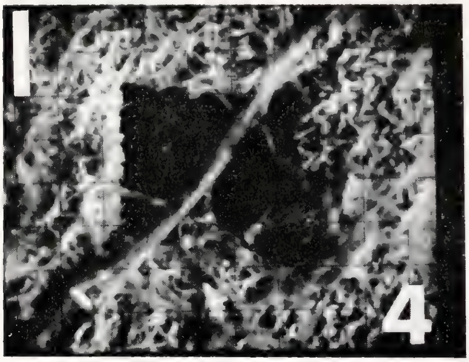
Piles of spicules were then digested on gold grids and the residue examined in the electron microprobe. Figures 2 and 3 show that carbon could be detected in these aluminum coated specimens. The carbon was restricted to those portions of the grid in which residue was visible either in the light-optics image or in the secondary electron scattering image shown in Figure 2. This residue contained large quantities of other elements because the non-volatile digestion products include fluorides of Na, K, Al, and traces of several other elements previously reported (Schwab and Shore, 1971).

Encouraged by the presence of carbon, bulk preparations were made of HF resistant material. The residue that was insoluble in 1 N HF was collected and washed in 1 N HF on a teflon filter. This residue was found in the electron microprobe to contain regions of high carbon content as illustrated by Figure 4 and 5. A point count of this region indicated the presence of up to 40% C. The electron scattering image confirmed that this region was an irregular pile of filamentous material with the dimensions of axial filaments or clusters of these. Individual threads about the diameter of axial filaments may be seen in Figure 4 in the region giving this high carbon content. A second bulk preparation was made to provide material for quantitative organic analysis. From 6.89 g of purified spicules, the total non-volatile residue after digestion in 12 N HF, collected on teflon, was 596 mg. Of this, the fraction insoluble in 1 N HF, collected on a teflon filter, was 12.4 mg. Determination of protein by the method of Lowry *et al.*









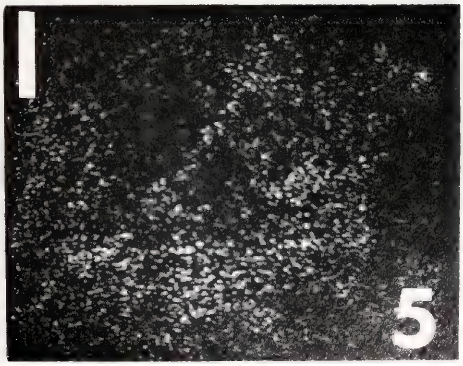


FIGURE 1. Secondary electron image of a single, partly etched style with HF resistant axial filament, scale line = 20 μ.

FIGURE 2. Secondary electron image of spicule residue on gold 400 mesh grid.

FIGURE 3. Carbon x-ray image of the field shown in Figure 2, with superposed plot of intensity of these x-rays (ordinate *vs.* distance).

FIGURE 4. Secondary electron image of washed spicule residue on gold 400 mesh grid.

FIGURE 5. Carbon x-ray image of the field shown in Figure 4.

TABLE I

Mole per cent composition of axial filament protein

Residue	Mole%	Residue	Mole%	Residue	Mole%	Residue	Mole%
Asp/Asn	14	Ala	6.8	Pro	5.0	His	2.7
Gly	11	Ser	6.3	Phe	5.0	Arg	2.8
Val	10	Leu	6.3	Lys	3.7	Tyr	1.4
Glu/Gln	9	Ile	5.9	Cys	2.9		
		Thr	5.5	Met	2.9		

(1951) on ¼ of this filter gave a color equivalent to 900 μg protein. That indicates the presence of about 3.6 mg protein in the 12.4 mg residue for about 29% protein. This 12.4 mg residue had the characteristic lavender color we have noted before in preparations of spicule residue. In HF or upon drying from HF, the residue is lavender. Water washing changes the color reversibly to a straw tan.

The results of the determinations of amino acid composition are summarized in Table I. When the initial separations were made using the Lowry value for protein content, the quantity of amino acids recovered was so far below that estimated that the chromatograph could not be used for a quantitative indication of the amino acid composition. A pair of determinations was then made using larger quantities of material. The most reliable estimate of the values for methionine and cysteine are believed to be those based on the pre-oxidized sample from which methionine is recovered as the sulfon and cysteine as cysteic acid. The paired values to other amino acids are within one mole per cent of each other, except as expected for methionine and cysteine. The major components of axial filament protein are aspartic (including asparagine), glycine, valine, and glutamic (including glutamine). There is no detectible hydroxyproline or hydroxylysine. Tryptophane would have been destroyed by the hydrolysis so it remains totally unestimated.

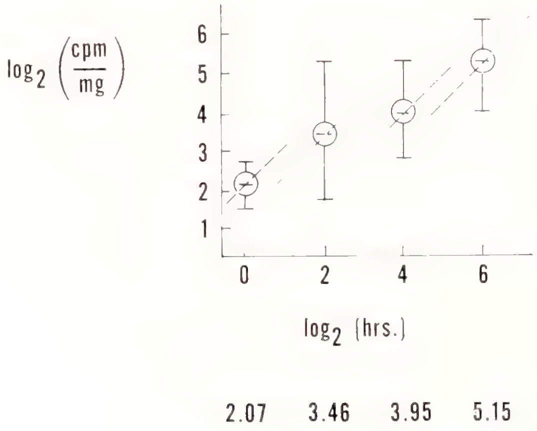


FIGURE 6. Time course of 3H-leucine incorporation into bulk protein for fingerlets of *Hymeniacidon sinapium.*

Incorporation studies

The incorporation of tritium from l-leucine into protein in sponge tissue occurs at 18° C. A determination of that incorporation as a function of duration of incubation is presented in Figure 6. The accumulation of incorporated radioactivity increases with time at least up to 64 hours under the conditions of incubation. The rate of accumulation decreases with increasing time in the presence of radioactive precursor. This may be seen from the slope in the figure. The dotted lines indicate the incorporation required for constant incorporation rate. However, each of the time points (except the 64 hour point) was based on both early and late replicate fingerlets. Furthermore, the rate of incorporation for *both* early and late 1-hour incubations is greater than the mean incorporation rate for 4-hour incubations. Therefore, this falling incorporation rate is not the result of a falling biological synthesis rate but rather is more likely the result of a falling specific activity of precursor pools. Thus the conditions of incorporation underestimate but do not inhibit protein synthesis during prolonged incorporation into intact sponge fingerlets.

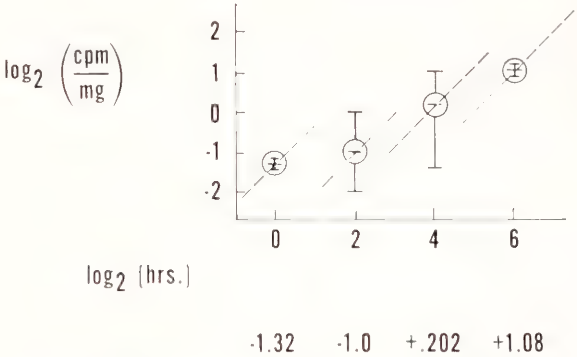


FIGURE 7. Time course of 3H-leucine incorporation into axial filament for fingerlets of *H. sinapium.*

Some incorporation has been detected into what is believed to be axial filaments. Tritium from leucine, equal to a small fraction of that incorporated into bulk protein, is incorporated into the HF non-volatile residue of spicules. The procedure of HF digestion leaves the axial filament morphologically without change, but completely removes organic matter from the spicule surface prior to HF digestion. The time course of incorporation of tritium into this non-volatile spicule residue in intact sponge fingerlets is shown in Figure 7. The incorporation increases with time. The rate of incorporation appears to be gradually decreasing with duration but there is no significant correlation between the rate of incorporation and the duration of the pre-incorporation incubation.

Finally the incorporation of tritium into the HF-insoluble residue was measured for cell suspensions. The data in Table II show the radio-activity that is not removed from spicules by repeated nitric acid digestion in the presence of added "carrier" spicules, nor subsequently made soluble by digestion and washing on a filter with 1 N HF. Statistically significant incorporation occurred in both "intermediate" and "dense" cell preparations. No incorporation was detected in either the "whole" or the "light" cell preparation.

Discussion

These data on the composition of axial filament are direct evidence that protein is a constitutent of the axial filament of silicious sponge spicules. This confirms the conclusion of many workers based on a large body of indirect evidence. The most important early work was Bütschli's exhaustive testing of spicule residues and microscopic observation of axial filament, in various reagents (Bütschli, 1901). Others have noted the presence of an axial filament resistant to digestion in HF (deLaubenfels, 1932; Lévi, 1963; Minchin, 1909; and Travis, Camille, Bonar and Glimcher, 1967). The most convincing evidence of a substantial protein component in axial filament comes from Garrone (1969). By digestion in selected enzymes he showed that a protease-sensitive material is required for the structural integrity of the axial filament. This enzymatic evidence that protein was an important component of the axial filament of sponge macroscleres provided no estimate of the fraction of axial filament that was protein. We had direct evidence from combustion and gas chromatography that there was sufficient carbon in the residue from HF digestion of spicules to provide a structure of the size of the axial filament and a composition of 40% C as it would be if solely protein or carbohydrate (Schwab and Shore, 1971). I, therefore, wished to determine the position in the spicule and the chemical form of that carbon.

Table II

Radioactivity of axial filament fraction after 3-H-leucine incorporation by cell-suspension preparations of Hymeniacidon sinapium. *Axial filament fraction was the residue after (a) digestion in 3 changes of hot conc. HNO_3 in the presence of "carrier" spicules, (b) digestion in 1 N HF, (c) membrane filter washing in 1 N HF. Radio-activity determined on filters in toluene-flours by liquid scintillation.*

Cell preparation	Incubation condition		
	With 3-H-Leucine	IOSW alone	Difference
Light	17.7 ± 0.72	17.6 ± 0.72	0.1 ± 1.4
Intermediate	23.8 ± 0.96	15.1 ± 0.60	8.7 ± 1.4
Dense	20.4 ± 0.80	13.4 ± 0.56	6.8 ± 1.3

From earlier analyses (Schwab and Shore, 1971) we inferred that carbon was present in the bulk axial filament residue, enough to provide up to 40% of the axial filament as protein or carbohydrate. From the microprobe data presented here, we may infer that the only detectable concentration of carbon occurs in the spicule residue in the position of individual axial filaments. From the Lowry assay datum we may infer that some protein is present in that residue, perhaps as much as 30% of the residue. From the amino acid analysis data we may infer that a material with the composition of protein is present in the residue and represents upwards of 8% of the Lowry-positive material, or upwards of 2% of the HF-insoluble residues. We have thus set a lower limit on the proportion of protein in the axial filament. The conclusion, that upwards of 2% of the axial filament is protein, is consistent with Garrone (1969). There is an apparent discrepancy between this conclusion and that of Drum (1968) and of Travis *et al.* (1967), namely, they conclude that the axial filament is carbohydrate. The relative proportions of protein and carbohydrate in axial filament and their manner of combination remain as yet unknown.

A large body of theory exists dealing with the manner in which specific protein synthesis is regulated (see for example the volume summarized by Lengyel (1969). In order to bring this theory to bear on the control of spicule growth it would be sufficient to show that specific protein synthesis is associated, in a non-trivial way, with spicule growth or more strongly, with silicious deposition in spicules. The data presented above support the conclusion that there is an association in both time and place between on the one hand, the incorporation of a protein precursor into some material associated with protein and, on the other, the deposition of apparently silicious material. The deposition is at least sufficient to protect the protein and the incorporated label from digestion by boiling nitric acid.

There remain yet to be determined the degree to which the label in the axial filament is actually in leucine, in protein, in a specific protein, or in one required for silicious deposition. This last point is the thrust of experiments to be undertaken next.

We have thus provided a procedure by means of which we may now ask about the relationship between protein synthesis (and incorporation into axial filament) and silicious deposition (in spicules) in the analysis of sponge growth and development. The present experiments thus make possible the experimental posing of questions which are formulated in terms of mechanisms regulating protein synthesis and which bear directly on the mechanism of regulation of silicious deposition.

It is a pleasure to express my appreciation for assistance with the technical procedures to Dr. Bigelow, University of Michigan, Ann Arbor, for the electron microprobe data, and to Miss Sandy Swartz and Dr. R. Serriano, Medical College of Ohio at Toledo, for the amino acid analysis data. This work was supported by a grant from Owens-Illinois Corp., through the Department of Fundamental Research, Dr. James Scala, Director.

SUMMARY

1. A substantial portion of the axial filament of the spicules of silicious sponges is protein.

2. The axial filament protein lacks the hydroxylated amino acids that are characteristically abundant in collagen.

3. It is feasible to follow the course of silicious deposition in spicules using C^{14} or H^3 in protein precursors.

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MITOTIC CYCLES IN OXYGEN UPTAKE AND CARBON DIOXIDE OUTPUT IN THE CLEAVING FROG EGG

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The early divisions in the fertilized frog egg (*Rana playtrrhina*) appear to be accompanied by a faint cycle in the rate of oxygen uptake, best described by an approximation to a sine curve; at each division the division furrow makes its appearance shortly after the respiratory rate has passed a minimum (Zeuthen, 1946).

In the modified Cartesian diver then used the gas phase was only slightly larger than the egg itself. This served the purpose of obtaining pressure changes fast enough to permit precise readings. The price paid was that respiratory CO_2 could not be absorbed chemically. However, as the purpose was to arrange that the respiring egg should take its oxygen from the diver's gas phase and deliver metabolic carbon dioxide to the diver's water phase, use was made of the large difference in solubility of the two gases in water. Thus, in the "frog-egg diver" the ratio between water and gas phases was high enough to secure effective, though not absolute separation in the diver's space between the two gases. Assuming that the period in O_2-uptake is respiratory and matched by a similar period in CO_2-output, Linderstrom-Lang (1946) calculated that probably the true biological cycle had been measured with slight delay—reflecting diffusion distances in the system—and with some damping—reflecting diffusion *and* incomplete separation of O_2 and CO_2.

Measurements of oxygen uptake already referred to can be performed with the same precision with divers having much larger gas space than used before, provided that a more sensitive manometer is adopted. Using divers with an expanded gas phase offers the further opportunity of reducing the ratio between the diver's water and gas phases to the limit that the major fraction of metabolic CO_2 ends up in the gas phase, provided the flotation medium and neck fluid are kept acidic. Pressure changes measured under these conditions reflect the difference between oxygen taken up from and carbon dioxide liberated to the diver's gas phase. On the other hand, if the flotation medium and neck fluid are alkaline, CO_2 becomes chemically absorbed. Consequently, CO_2 can be measured as the difference between results obtained under comparable conditions with and without CO_2-absorption.

Experiments of this sort were performed previously with marine eggs (Zeuthen, 1949; Frydenberg and Zeuthen, 1960). Presently we have adapted the same principle for work with single, cleaving frog eggs.

MATERIALS AND METHODS

Eggs of *Rana oxyrrhina* and of *Rana platyrrhina* were used in this study. They were artificially fertilized in $\frac{1}{10}$ Standard Solution (3.5 g NaCl, 0.05 g KCl, 0.1 g $CaCl_2$, 0.2 g $NaHCO_3$, 1 liter H_2O, pH 6.7 (Rugh, 1952)).

For measurement of gaseous exchanges in single eggs we used a stoppered Cartesian diver in conjunction with a sensitive manometer (Zeuthen, 1955). The rate of oxygen consumption ($\Delta O_2/\Delta t$) was measured with a diver having a gas volume of 12.3 μl and a fluid volume of 10 μl. The flotation medium was 0.02 N NaOH. Measurements of the oxygen uptake minus the carbon dioxide output ($(\Delta(O_2 - CO_2))/\Delta t$ were carried out with a diver having a gas volume of 53 μl and a fluid phase of 16 μl. In this case the flotation medium was $\frac{1}{10}$ Standard Solution. The time for the appearance of the initial 5 cleavages was determined using a horizontal microscope (15×). The gas volume (V) (atmospheric air) of each diver was measured by use of a Holter breaking pipette (Holter, 1943) as described by Zeuthen (1955). Gas exchanges were calculated by use of the equation in the same paper. The sensitive burette manometer was read with an accuracy of ±0.2 mm. For measurements of $\Delta O_2/\Delta t$, changes in burette readings over the measuring period were around 40–50 mm, corresponding to a reading error of only $\pm\frac{1}{2}\%$. For measurements of $\Delta(O_2 - CO_2)/\Delta t$, pressure changes per measuring period were smaller, *viz.* 8–12 mm, and the reading error about ±3%. Inspection of the curves presented in this paper suggests that the precision with which gaseous exchanges are measured approaches the precision with which pressure changes are read.

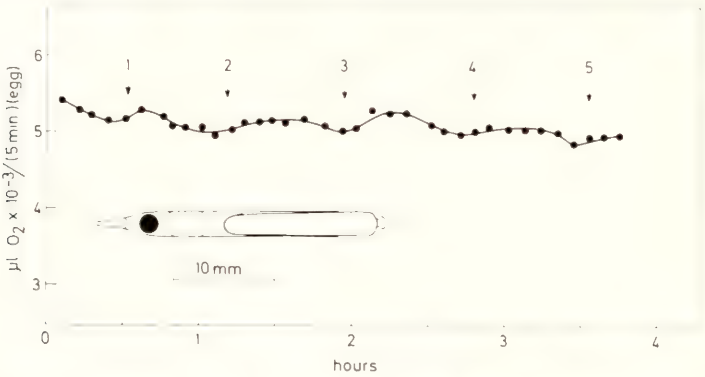

FIGURE 1. Rates of oxygen uptake ($\Delta O_2/\Delta t$) in a single egg of *Rana platyrrhina*. Arrows indicate when cleavages 1–5 begin. Insert shows diver used; it floats "egg-down."

RESULTS

For measurements of $\Delta O_2/\Delta t$, a single egg of *Rana platyrrhina* fertilized in $\frac{1}{10}$ Standard Solution (minus the bicarbonate) was isolated in the stoppered diver (gas volume 12.3 μl) shown in Figure 1. The curve shows ΔO_2/5 min against time. The arrows indicate when cleavages 1–5 begin. The observations are fitted by a periodic curve. There is one wave per mitotic cycle, the height of which (from valley to top) is 3–5% of the overall rate (Table I), and the furrow is incipient when the rate of oxygen consumption is beginning to increase after having passed a minimum. Both observations confirm earlier results by Zeuthen (1946).

In a different study we measured $\Delta O_2/\Delta t$ through cleavage using eggs of *Rana oxyrrhina* in gradient ampulla divers (Hamburger and Zeuthen, in preparation).

TABLE I

Wave amplitudes

		2 cells	4 cells	8 cells	16 cells
$\Delta O_2/\Delta t$		4.0%	3.4%	5.1%	2.8%
$\Delta(O_2 - CO_2)/\Delta t$	A	15%	20.5%	21%	27%
	B	20%	20%	28%	
$\Delta CO_2/\Delta t$	A	11%	15%	16%	
	B	11.5%	11.5%	18%	

While observations for the latter species spread somewhat more around the curves than in the present Figure 1, the rhythm in $\Delta O_2/\Delta t$ seems the same in both species. Therefore, direct comparison of results obtained with the two species of eggs is permissible.

Measurements of $\Delta(O_2 - CO_2)/\Delta t$ for two fertilized eggs (*Rana oxyrrhina*) studied through the first 4–5 cleavages are shown in Figure 2. We used a stoppered diver with a gas volume of 53 μl and a water phase of 16 μl (shown). The flotation medium was $\frac{1}{10}$ Standard Solution (exp. A), or this medium omit-

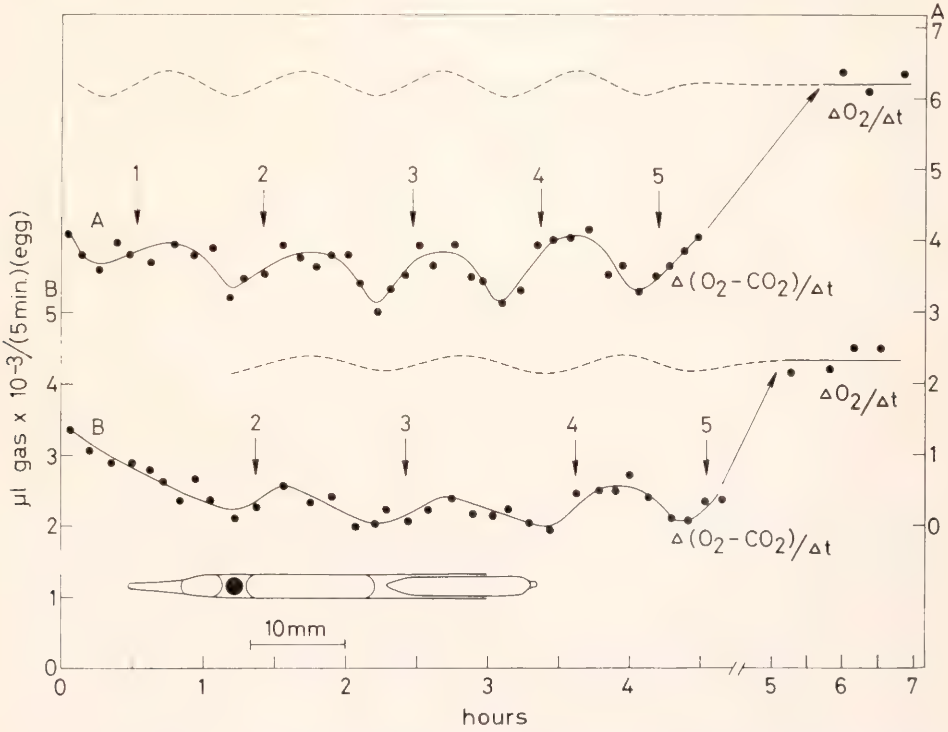

FIGURE 2. Rates of gaseous exchanges in two single eggs (A and B) of *Rana oxyrrhina*. The fully drawn curves show $\Delta(O_2\text{–}CO_2)/\Delta t$ and $\Delta O_2/\Delta t$ measured, respectively, in the intervals O–5 and 5–7 hours. The dashed curves show extrapolated values for $\Delta O_2/\Delta t$. Arrows indicate when cleavages 1–5 begin. Insert shows diver used; it floats "egg-up."

ting the bicarbonate (exp. B). At the end (4.5 hours) of the measurements of $\Delta(O_2 - CO_2)/\Delta t$ the flotation medium and diver's neck fluid were replaced with 2/100 N NaOH, so that measurements of $\Delta O_2/\Delta t$ could be made between 5.5 and 7 hours.

The curves for $\Delta(O_2 - CO_2)/\Delta t$ resemble those for $\Delta O_2/\Delta t$ in that there is one wave per mitotic cycle and furrow formation comes at a time when the rate of gaseous exchange is increasing. However, these curves show more cyclicity as demonstrated by Table I. Differences, to be read on the ordinates, between pairs of curves for $\Delta O_2/\Delta t$ (here dashed in at proper levels and in proper relation to the cleavage cycle) and $\Delta(O_2 - CO_2)/\Delta t$ represent carbon dioxide liberated to the diver's gas space.

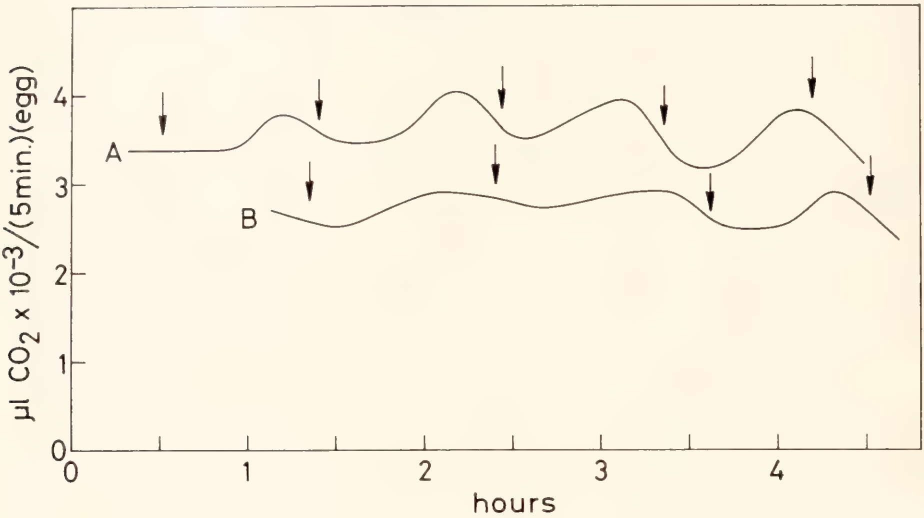

FIGURE 3. Rates of carbon dioxide production ($\Delta CO_2/\Delta t$) calculated on basis of the curves shown Figure 2. Arrows indicate when cleavages 1–5 (A) and 2–3 (B) begin.

The total amount of carbon dioxide liberated by the egg was calculated on the basis of two assumptions: (1) The diver is tight to CO_2, and (2) the total volume of water in the diver (always including the egg water) serves as a dissolving space for CO_2. These assumptions are discussed at length elsewhere (Hamburger and Zeuthen, in preparation) and seem to be justified. We first calculated $\Delta CO_2^{air}/\Delta t$, *i.e.*, CO_2 liberated to the diver's air space, as represented by the area, over a narrow segment of the abscissa, between curve $\Delta(O_2 - CO_2)/\Delta t$ and the corresponding curve $\Delta O_2/\Delta t$ (dashed). The amount of CO_2 liberated by the egg per unit time, $\Delta CO_2/\Delta t$, approximates

$$\Delta CO_2/\Delta t = \Delta CO_2^{air}/\Delta t(k + 1),$$

in which k is the ratio between water and air volumes in the diver times the absorption coefficient for CO_2 in water at the temperature of the experiment; k is derived from linear measurements of the compartments of the diver, Figure 2. In

Figure 3 are shown curves (A, B) thus calculated for $\Delta CO_2/\Delta t$. They show a reproducible rhythm in CO_2-liberation, with small bursts $\frac{1}{4}$ of the cycle time before appearance of the division furrows, and corresponding dips are seen $\frac{1}{4}$–$\frac{1}{3}$ of a cycle after this event, thus between the times of maximum and minimum rates of O_2 uptake (Fig. 1). Percentage-wise, rate changes in CO_2 evolution are more expressed than are the fluctuations in ΔO_2: 11–18% *versus* 3–5% (Table I). Values for RQ were calculated (Fig. 4) from the curves of Figure 2. Respiratory quotients were found to fluctuate by 0.10–0.15 around the mean (dashed curve) and almost parallel to the values for CO_2. The mean values (dashed curve) for RQ were 0.58 (A) and 0.65 (B).

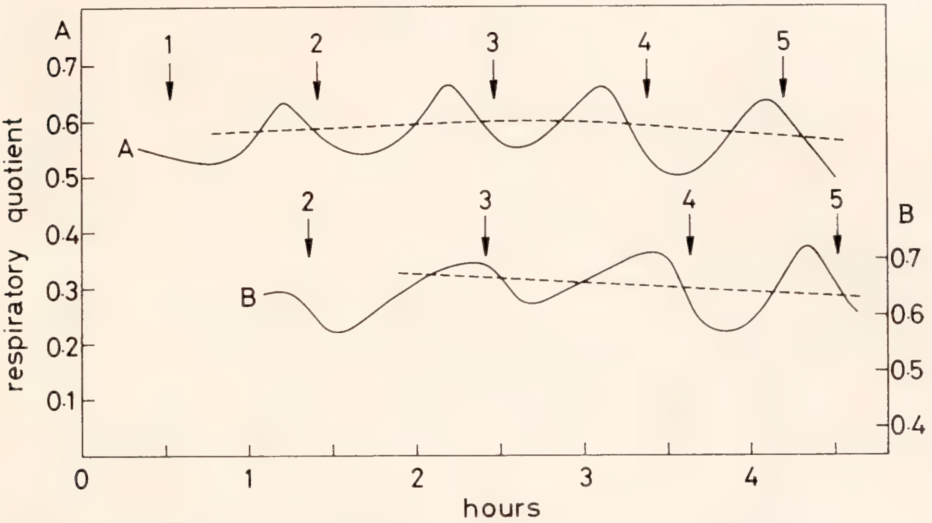

FIGURE 4. Respiratory quotients calculated on basis of values for $\Delta O_2/\Delta t$ (Figs. 1 and 2) and $\Delta CO_2/\Delta t$ (Fig. 3). Arrows indicate when cleavages 1–5 begin.

DISCUSSION

Below we discuss measurements of rates of oxygen uptake ($\Delta O_2/\Delta t$), of rates of gaseous exchanges when CO_2 is not chemically absorbed in the system ($\Delta(O_2 - CO_2)/\Delta t$), and of rates of carbon dioxide output ($\Delta CO_2/\Delta t$), obtained as the difference between results from the two first types of measurements. Finally, we shall discuss relations between uptake of O_2 and output of CO_2 in the cleaving frog egg.

We first discuss Figure 1, in which $\Delta O_2/\Delta t$ was measured. In this experiment diffusion distances were somewhat long in comparison to other experiments (Zeuthen, 1946). The egg center was separated from the gas phase by 2.17 mm, and the length of water column between the animal pole of the egg and the diver's gas phase was 0.9 mm. In the following we make use of Linderstrøm-Lang's mathematical considerations as they were applied to previous experiments of the type here performed, and particularly to his Table III (Linderstrøm-

Lang, 1946, page 242). If in our Figure 1 the egg's respiration was confined to a plane through the egg center and perpendicular to the diver's axis, faint periodic respiratory oscillations of the type perviously found (Zeuthen, 1946) would be picked up with 30% damping and with 6 min delay. On the other hand, if the egg's respiration was located in a plane at the animal pole of the egg (0.9 mm from diver's gas phase) actual respiratory oscillations in the egg would be only 6% damped, and they would be reflected at the level of the diver's air with a delay of only 3 min. Actual damping and delay in the present measurements may have been between the two sets of values, and probably closer to the latter than to the former. Wave amplitudes in Figure 1 average 4.2%. They are in the range (3.2–7.7%) and as near the mean (5.8%) of previous measurements (upper part of Table II in Zeuthen, 1946) as can reasonably be expected in view of small differences between the two sets of experiments, some of which have been mentioned.

We next discuss experiments in which CO_2 was not chemically absorbed in the diver's water phase, only partly held in physical solution. In two experiments (Fig. 2) we found expressed mitotic periodicity of $\Delta(O_2 - CO_2)/\Delta t$. Essentially, this must be referred to $\Delta CO_2/\Delta t$. In the two experiments CO_2 produced by the egg can be expected to distribute itself in the ratio 4:1 between the diver's air and water phases. This assumes that quasi-steady states pertained, which assumption derives support from comparison between the overall course taken by the curves in the present Figure 2 and in Linderstrøm-Lang's theoretical Figure 10 (Linderstrøm-Lang, 1946). Thus, what we have directly measured approximates $\Delta(O_2 - \frac{4}{5} CO_2)/\Delta t$. As demonstrated in the result section, $\Delta O_2/\Delta t$ and $\Delta CO_2/\Delta t$ both show oscillations. These must be somewhat damped and delayed relative to the true oscillations in the egg. From the calculations of Linderstrøm-Lang (1946, Table III and text page 235) we arrive at the conclusions that in our experiments (distance from egg-center to air $\leqq 1.3$ mm) damping of the wave amplitude may have been around 10% and the waves may have been measured with perhaps 3 minutes delay. We have not felt justified in applying such small corrections, and the following discussion is based on inspection of the primary curves.

The question may be raised if the rhythm in CO_2-output in part reflects that states of partial anaerobiosis recur periodically in elements of the egg remote from the diver's air space. We have discussed this issue elsewhere (Hamburger and Zeuthen, in preparation) and there reached the conclusion that this situation is not likely to pertain.

In conclusion, in the cleaving frog egg values for $\Delta O_2/\Delta t$ change slightly and rhythmically. We can observe only one respiratory wave per mitotic cycle, and fluctuations occur with an amplitude of only 4–6%: $\Delta O_2/\Delta t$ is minimal at or slightly before the appearance of each division furrow, it is maximal 20–25 min later, equal to 25–30 min before the appearance of the subsequent division furrow. Fluctuations in $\Delta CO_2/\Delta t$ are more pronounced. They occur with an amplitude in the order of 11–18%. We can account for only one wave in $\Delta CO_2/\Delta t$ per mitotic cycle; however, as these results are in terms of differences between two sets of measurements we should not exclude the possibility of further complexity in true curve shape. In our study $\Delta CO_2/\Delta t$ was maximum at or slightly before the onset of cytokinesis and minimum 20–30 min later. Thus, cyclic curves for $\Delta O_2/\Delta t$ and for $\Delta CO_2/\Delta t$ are shifted about one half cycle relative to each other.

We next consider the timing of respiratory oscillations with stages in the division cycle of the eggs. According to Brachet (1960) working with *Rana fusca* the incipient cleavage furrow is seen at telophase. In *R. nigromaculata* (Kubota, 1965) the furrow appears a little later than the phase when chromosomal vesicles fuse. The two statements essentially agree. They may cover timing differences, which maximally correspond to perhaps 10% of cell cycle time. (It is in view of this that the small delay—perhaps 5% of cycle time—with which we measured mitotic oscillations in $\Delta O_2/\Delta t$ and in $\Delta CO_2/\Delta t$ seems rather insignificant.)

If comparison between species of frog eggs is permitted we can suggest that maxima for rates of O_2-uptake and minima for rates of CO_2-output are seen towards the end of each interphase, and that rates of O_2-uptake decrease and of CO_2-output increase through each mitosis proper. Thus the rate of oxygen uptake is lowest and of carbon dioxide output highest around the end of each mitosis.

Respiratory quotients cycle by 0.10–0.15 around a mean which is 0.6 in experiment A and 0.65 in experiment B (Fig. 4). As the quotient is more influenced by the large variations in $\Delta CO_2/\Delta t$ than by the smaller changes in $\Delta O_2/\Delta t$, $\underset{\sim}{RQ}$ shows minima in late interphase and maxima at the end of each mitosis (Fig. 4).

The present observations for oxygen uptake through the egg's division cycle fully confirm earlier results by Zeuthen (1946), and they rule out the possibility mentioned by him that periodicities in oxygen uptake be only apparent and reflect fluctuations in the production of carbon dioxide—incompletely absorbed in those earlier studies. Moreover, there is good correspondence with earlier results by Holter and Zeuthen (1957) for the sea urchin *Psammechinus microtuberculatus.* They found minima in the rate of oxygen uptake at telophases, maxima at prophases.

There appear to be differences between small marine eggs and frog eggs with respect to carbon dioxide output during the early division cycles. In the course of the first 5–6 divisions in eggs of *Urechis caupo* and of *Dendraster excentricus,* Zeuthen (1949) and Frydenberg and Zeuthen (1960) did not find the cycle in carbon dioxide output which we here report for the cleaving frog egg, and in their discussion they raised the issue if in those eggs decarboxylating steps in respiration in fact function linearly during the early divisions. However, taking results for later cleavage stages into account they found this unlikely, and instead suggested that throughout cleavage, respiratory oxygen uptake and respiratory carbon dioxide output cycle in a parallel fashion and in time with the sequence of mitoses. They considered that the manometric measurements of respiratory CO_2-output might be complicated by cyclic changes in the amount of CO_2 chemically retained by the egg. Their results fit the idea that the eggs turn more acidic from prophase through telophase (respiration rate falls through mitosis proper) and again more alkaline during interphase (at the same time as respiration rate again increases). The present results with single frog eggs might fit this suggestion. However, increase in acidity during mitosis would be more expressed and so would reversal towards alkalinity during interphase.

SUMMARY

Rates of oxygen uptake and of carbon dioxide output of single cleaving frog eggs were measured at short intervals. A Cartesian diver modified for the purpose was used in conjunction with a sensitive manometer. Carbon dioxide outputs were measured as the difference between results obtained with and without absorption of CO_2 in the system.

For both measured parameters reproducible variations were found to accompany the mitotic cycle. Oxygen uptake occurred at minimal rate shortly before the beginning of each new division (cleavage furrow) and at maximal rate halfway between two maxima. Rates of carbon dioxide production varied in an inverse manner and at the same time were more pronounced. Consequently, RQ cycled regularly around a mean (0.6–0.7) and with an amplitude of about 0.1–0.15 units. RQ maxima were at 10–15 min before, minima at 10–20 min after the beginning of furrow formation.

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Volume 143 Number 1

THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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AUGUST, 1972

Marine Biological L
LIBRA
AUG 24 1972
Woods Hole, Mass.

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $5.00. Subscription per volume (three issues), $14.00, (this is $28.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. W. D. Russell-Hunter, Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between May 23 and September 1, and to Dr. W. D. Russell-Hunter, P.O. Box 103, University Station, Syracuse, New York 13210, during the remainder of the year.

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